Appendix C

EVIDENCE THAT HUMAN CARDIAC MYOCYTES DIVIDE AFTER MYOCARDIAL INFARCTION

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ABSTRACT

Background The scarring of the heart that results from myocardial infarction has been interpreted as evidence that the heart is composed of myocytes that are unable to divide. However, recent observations have provided evidence of proliferation of myocytes in the adult heart. Therefore, we studied the extent of mitosis among myocytes after myocardial infarction in humans.

Methods Samples from the border of the infarct and from areas of the myocardium distant from the infarct were obtained from 13 patients who had died 4 to 12 days after infarction. Ten normal hearts were used as controls. Myocytes that had entered the cell cycle in preparation for cell division were measured by labeling of the nuclear antigen Ki-67, which is associated with cell division. The fraction of myocyte nuclei that were undergoing mitosis was determined, and the mitotic index (the ratio of the number of nuclei undergoing mitosis to the number not undergoing mitosis) was calculated. The presence of mitotic spindles, contractile rings, karyokinesis, and cytokinesis was also recorded.

Results In the infarcted hearts, Ki-67 expression was detected in 4 percent of myocyte nuclei in the regions adjacent to the infarcts and in 1 percent of those in regions distant from the infarcts. The reentry of myocytes into the cell cycle resulted in mitotic indexes of 0.08 percent and 0.03 percent, respectively, in the zones adjacent to and distant from the infarcts. Events characteristic of cell division — the formation of the mitotic spindles, the formation of contractile rings, karyokinesis, and cytokinesis — were identified; these features demonstrated that there was myocyte proliferation after myocardial infarction.

Conclusions Our results challenge the dogma that the adult heart is a postmitotic organ and raise the possibility that the regeneration of myocytes may contribute to the increase in muscle mass of the myocardium. (N Engl J Med 2001;344:1750-7.)

MYOCYTE replication occurs in the failing human heart,[1] and this form of cell growth tends to compensate for the exhaustion of myocyte hypertrophy.[2] In chronic heart failure, myocytes at most double in size, and when this limit has been reached no further enlargement of the heart occurs.[2,3] For decades, it has been doubted whether the heart can grow by multiplication of myocytes.[4] The demonstration that the adult human brain contains a population of cells that are able to regenerate neurons[5] has not prompted a comprehensive reexamination of the notion that the heart is a postmitotic organ,[6] even though cardiac endothelial cells, smooth-muscle cells, and fibroblasts are known to proliferate.[3,4] Recently, a myocyte mitotic index (the ratio of the number of nuclei undergoing mitosis to the number not undergoing mitosis) of 0.015 percent was measured in explanted hearts from patients in the terminal stages of cardiac decompensation.[1] The importance of these results was questioned on the assumption that this level of myocyte replication has no clinical significance.[4] In the absence of supporting evidence, it has been claimed that rates of myocyte proliferation ranging from 0.05 to 0.1 percent would be required for meaningful therapeutic reconstitution of damaged myocardium.[4]

Although a mitotic index of 0.015 percent, if sustained, could result in the formation of 100 g of myocardium in less than three months,[1] the fraction of mitotic myocytes in patients with late cardiac failure may reflect the ultimate growth reserve of this cell population. The mechanical overload in a disease that lasts several years may progressively exhaust the replicative capacity of myocytes. Cells cannot divide indefinitely. In contrast, after extensive myocardial infarction, abrupt increases in the need for growth may cause more myocytes to reenter the cell cycle than during chronic heart failure. To test this hypothesis, we determined the percentages of cycling myocytes and the mitotic indexes in patients who had died within a short time after extensive myocardial infarction. Cycling myocytes were identified by the expression of Ki-67 in nuclei. This nuclear protein is associated only with cell division.[7,8] Although the function of Ki-67 is not clear, it appears to promote cell proliferation by interfering with the binding of p53 to DNA.[9] To avoid errors in the identification of myocytes, we assessed mitotic divisions of myocyte nuclei by confocal microscopy of immunolabeled cell cytoplasm.[1,3] The measures of cell regeneration were determined separately

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in areas bordering on and distant from the infarcts. To investigate characteristics of cell division in vivo, we searched for the formation of the mitotic spindle by microtubules[10] and of the contractile ring by actin accumulation,[11] as well as karyokinesis and cytokinesis.

METHODS

Patients

Thirteen hearts were obtained from patients who had died 4 to 12 days after myocardial infarction. There were seven men and six women, with a mean (±SD) age of 64±15 years. As previously described,[12] we obtained 10 control hearts at autopsy from five men and five women, with a mean age of 61±20 years, who had died from causes other than cardiovascular disease. Infarct size was determined by measuring the area of grossly detectable necrotic myocardium within the left ventricular free wall; this was expressed as a fraction of the total area.[13] The rest of the left ventricular free wall was divided into three equal parts, which were defined as the border zone, the intermediate region, and the distant myocardium. Since the average size of the infarct was 35 percent, the average size of each area of viable tissue was nearly 20 percent of the left ventricular free wall. Samples were obtained only from the border zone and the distant myocardium. Tissue specimens from comparable areas of noninfarcted control hearts were examined.

Confocal Microscopy and Immunocytochemical Analysis

The specimens were fixed in 10 percent phosphate-buffered formalin and embedded in paraffin. The histologic sections were stained with propidium iodide (10 μg per milliliter) and antibody to α-sarcomeric actin (clone 5C5, Sigma), diluted 1:20 in phosphate-buffered saline. For identification of mitotic spindles, the samples were exposed to a mouse monoclonal antitubulin antibody (Zymed). Fluorescein isothiocyanate–conjugated antimouse IgG was used as a secondary antibody. For the detection of Ki-67, the samples were exposed for one hour at 37°C to a mouse monoclonal antibody against Ki-67 (clone MIB-1, Diagnostic Biosystems), diluted 1:40 in phosphate-buffered saline.[7] Fluorescein isothiocyanate–conjugated antimouse IgG was again used as a secondary antibody. Subsequently, specimens were processed for confocal microscopy[1,3] and examined with a confocal microscope (MRC-1000, Bio-Rad).

Statistical Analysis

The numbers of myocyte nuclei labeled by Ki-67 were determined by evaluating approximately 3000 nuclei in the border zone and 9000 to 11,000 nuclei in the distant myocardium of each infarcted heart. Approximately 100,000 to 125,000 nuclei were evaluated in each control heart. The numbers of myocyte nuclei undergoing mitosis were determined by evaluating an average of 80,000 nuclei in the border zone and 104,000 nuclei in the distant myocardium of each infarcted heart. The values in corresponding anatomical areas of the control hearts were 100,000 and 110,000. Sampling for mitoses was larger than sampling for Ki-67 because of the lower frequency of mitoses in the myocardium. A total of 1165 Ki-67–positive myocyte nuclei was counted in infarcted hearts. This yielded an overall sampling error of 2.9 percent (the sampling error equals the square root of n divided by n, where n equals the total count). The number of myocyte nuclei undergoing mitosis was 590, reflecting a 4.1 percent sampling error.[14] These values are less than the biologic variability among humans, which is at least 20 percent.[14] Since the numbers of Ki-67–labeled nuclei and mitotic images were similar in these two control regions analogous to border and distant myocardium in infarcted heart, separate measurements were combined to generate a single value. The results are presented as means ±SD. The significance of the differences was determined with the use of Student's t-test for comparisons of two values and the Bonferroni method for multiple comparisons.[15]

RESULTS

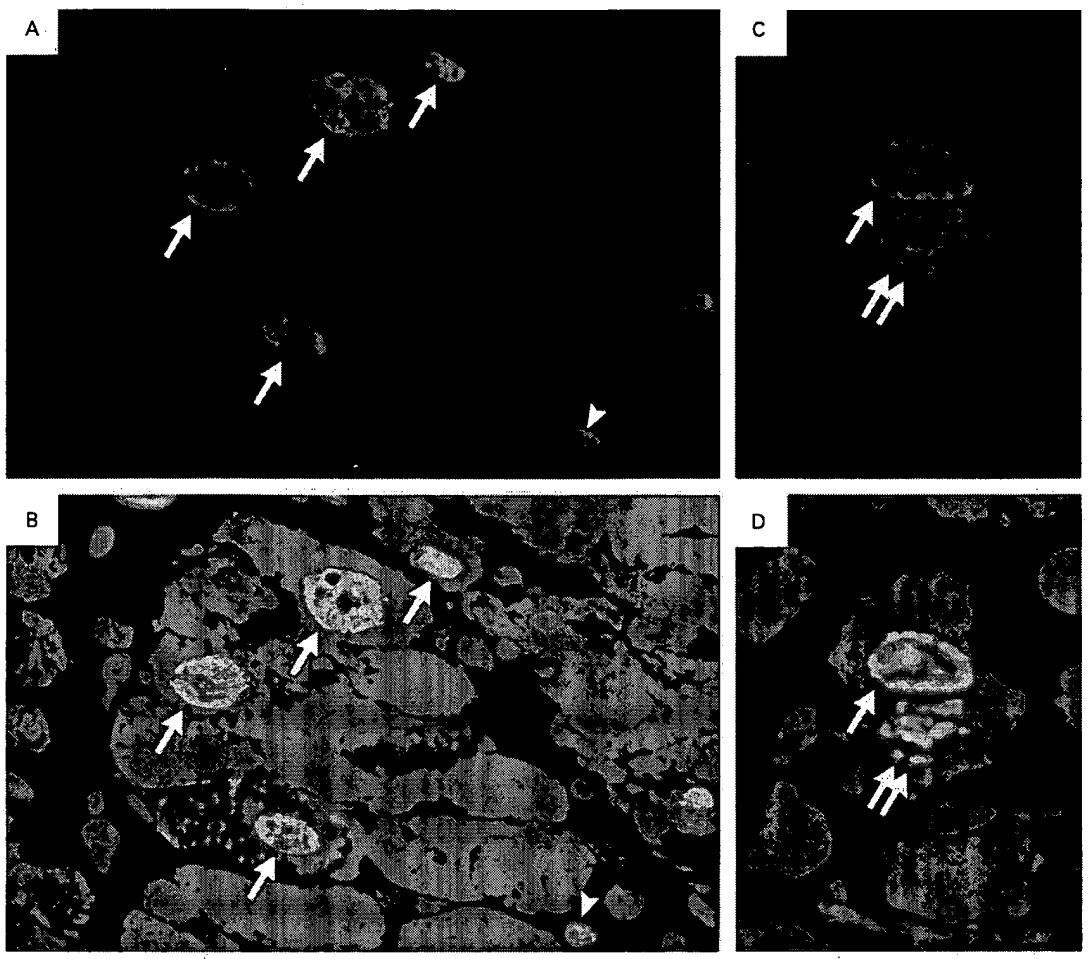
Patients

The hearts of the 13 patients with myocardial infarction were obtained 7 to 17 hours after death. Coronary atherosclerosis was severe and affected the left and right coronary arteries in all cases. Myocardial infarction consistently involved the anterior and inferior aspects of the left ventricle and was associated with cardiac rupture in three subjects. The size of the infarct ranged from 26 to 44 percent, averaging 35±7 percent. In three cases, an old fibrotic infarct was noted, and foci of replacement fibrosis and areas of interstitial fibrosis were identified in all three of these hearts. None of the patients had a history of systemic hypertension or diabetes. Two of the 13 patients had been treated with thrombolytic agents. The average weight of the hearts with myocardial infarction was 497± 129 g, and that of the control hearts was 361±51 g ($P=0.005$). The control hearts were from subjects of similar age who did not have primary heart disease or major risk factors for coronary artery disease, including hypertension, diabetes, obesity, and severe atherosclerosis. Autopsy and histologic examination of all organs ruled out the presence of diffuse metastatic malignant neoplasms and chronic inflammation. Six patients died from acute trauma, one from gastrointestinal hemorrhage, two from cerebral hemorrhage, and one from pulmonary thromboembolism.

Ki-67 Labeling and Mitotic Index

Ki-67 is a nuclear antigen expressed in all phases of the cell cycle except G_0.[7] Ki-67 is apparent mainly in the late S phase, increases further in G_2, persists during prophase and metaphase,[7] and decreases in anaphase and telophase. Ki-67 is preferable to thymidine, bromodeoxyuridine, and proliferating-cell nuclear antigen for labeling, because it is not involved in DNA repair.[16] Expression of Ki-67 is a requirement for cells to traverse the cell cycle and undergo cell division.[7,8] All types of proliferating human cells express Ki-67.[16,17] Ki-67 was measured in myocyte nuclei of control and infarcted hearts by confocal microscopy (Fig. 1A, 1B, 1C, and 1D).[1,3,18,19] In comparison with myocytes from normal hearts, the number of Ki-67–positive nuclei in myocytes from hearts with myocardial infarction was 84 times as high in samples from the border zone and 28 times as high in samples from the distant myocardium ($P<0.001$ for both comparisons) (Fig. 1E).

Although the expression of Ki-67 in myocytes after infarction by itself challenges the assumption that the heart is a postmitotic organ,[4,6,20] we found further evidence of myocyte division. During mitosis, microtubules form the mitotic spindle, allowing each chromatid to be pulled toward the spindle pole by the kinetochore microtubules. This process occurs in anaphase and lasts only a few minutes.[21] The arrange-



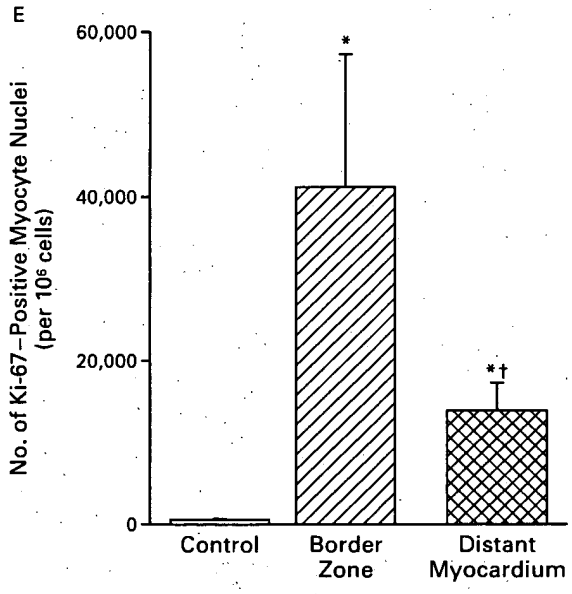


Figure 1. Ki-67 Labeling of Cycling Myocytes in an Infarcted Heart.

In Panels A and C, green fluorescence documents localization of Ki-67 in nuclei (arrows and arrowhead). In Panels B and D, red fluorescence shows staining of myocyte cytoplasm by sarcomeric α-actin antibody, and bright fluorescence shows labeling of myocyte nuclei (arrows) and nonmyocyte nuclei (arrowhead) by a combination of propidium iodide and Ki-67. Ki-67 labeling of a myocyte nucleus in metaphase is evident in Panels C and D (double arrows). Panels A and B show cells from the border zone, and Panels C and D cells from the distant myocardium. (Panels A, B, C, and D, ×800.) Panel E shows the effects of infarction on the mean (±SD) number of Ki-67–labeled myocyte nuclei. The asterisks indicate $P<0.001$ for the comparison between the infarcted hearts and the control hearts; the dagger indicates $P<0.001$ for the comparison between the distant myocardium and the border zone in the infarcted hearts.

ment of microtubules in the mitotic spindle of dividing myocytes was detected on microscopical examination (Fig. 2). In addition, accumulation of actin and its assembly in the contractile ring were identified with the use of sarcomeric α-actin antibody (Fig. 3). Myocyte division was in the process of completion and actin was condensed in a narrow region, delineating a groove between the two forming daughter cells. Images of nuclear mitotic division (Fig. 4) and cytokinesis (Fig. 5) were also obtained, strengthening the notion that Ki-67 labeling in nuclei represents multiplying myocytes.

As a direct quantitative estimate of the extent of myocardial repair, we calculated a myocyte mitotic index. Staining with antibody to sarcomeric α-actin is specific for I bands of cardiac and skeletal muscle cells and does not affect other actin isoforms.[22] Therefore, the distinction between myocyte and nonmyocyte nuclei is extremely simple: interstitial cells are not stained by α-sarcomeric actin, and only their nuclei can be seen on staining with propidium iodide (Fig. 2D, 4A, 4B, 4C, and 5B). The same approach was used for Ki-67 labeling (Fig. 1B and 1D). Myocyte mitotic indexes are shown in Figure 6. In comparison with normal hearts, hearts with myocardial infarction have 70 times as many myocytes undergoing mitosis in the border zone and 24 times as many in the distant myocardium ($P<0.001$ for both comparisons). The fact that the value was 2.9 times as high in the border zone as in the distant myocardium ($P<0.001$) is consistent with the higher level of Ki-67 expression in the border zone.

DISCUSSION

Our results indicate that the adult heart has a subpopulation of myocytes that are not terminally differentiated; these myocytes evidently reentered the cell cycle and underwent nuclear mitotic division early af-

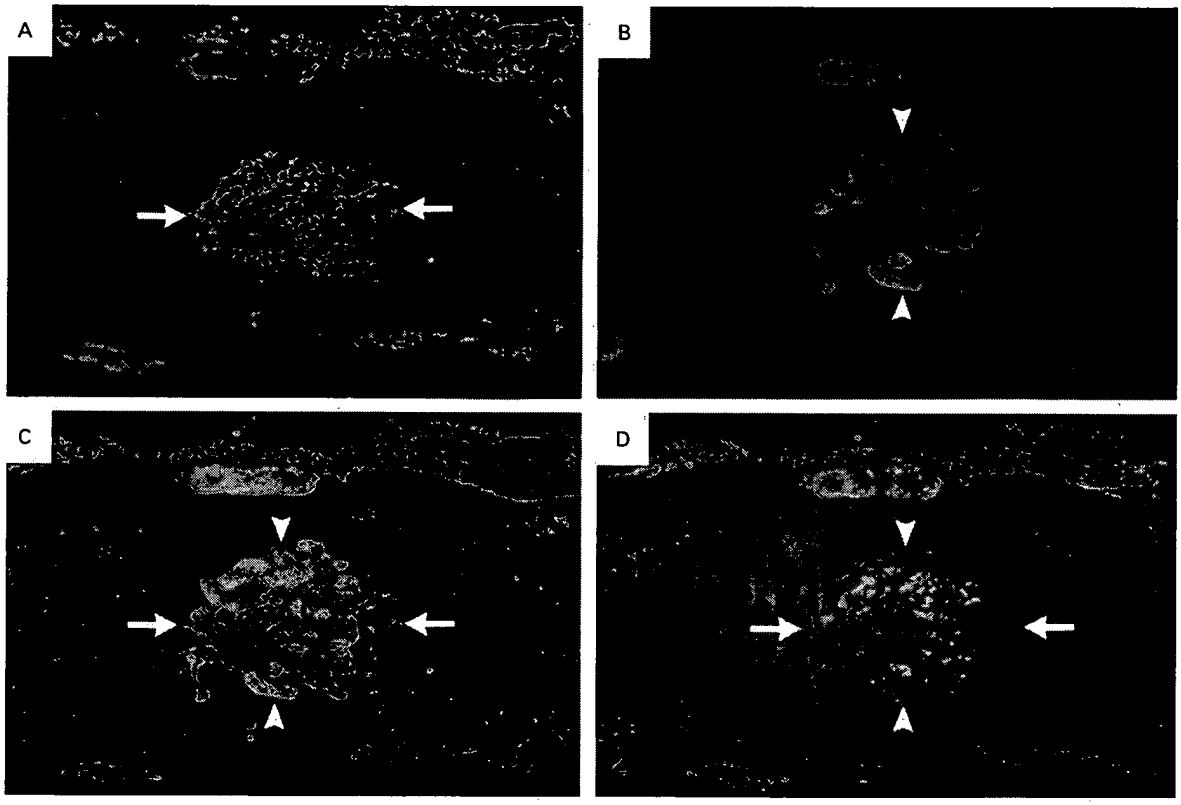


Figure 2. Identification of Mitotic Spindles in Dividing Myocytes from Infarcted Hearts (×2000).

In Panel A, blue fluorescence indicates the organization of tubulin in the mitotic spindle (arrows). Panel B depicts a nucleus in metaphase, indicated by the green fluorescence of propidium iodide (arrowheads). In Panel C, green and blue fluorescence shows the combination of tubulin and metaphase chromosomes (arrows and arrowheads). Panel D shows staining of myocyte cytoplasm by antibody against sarcomeric α-actin (red fluorescence), tubulin labeling (blue fluorescence), and chromosomes in metaphase (green fluorescence) (arrows and arrowheads).

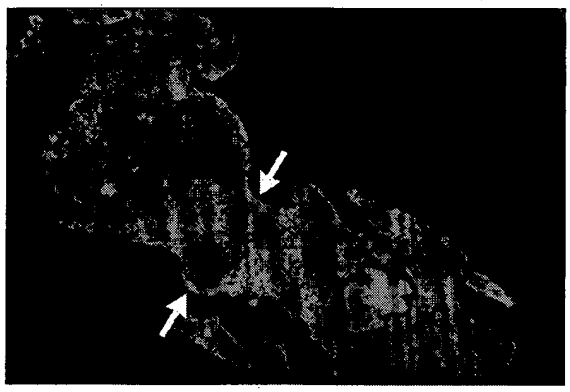


Figure 3. A Myocyte in the Process of Cytokinesis.
Accumulation of actin (arrows) in the region of cytoplasmic division and cell separation is shown; red fluorescence shows staining of myocyte cytoplasm by antibody against sarcomeric α-actin, and green fluorescence shows propidium iodide labeling of chromosomes (×2000).

ter infarction. The number of cycling myocytes was significantly larger in the zone bordering the infarct than in the distant myocardium. In laboratory animals, conditions that mimic coronary artery disease are characterized by DNA replication and myocyte division.[3,23] These responses peak 7 to 14 days after coronary-artery restriction and decrease with time.[23] A similar phenomenon may occur in humans, suggesting that prolonged heart failure may progressively affect the mitotic activity of myocytes. Multiplication of myocytes is markedly attenuated as the length of time after myocardial infarction increases.[1]

DNA synthesis in myocyte nuclei has been measured experimentally on the basis of incorporation of nucleotides such as [^{3}H]thymidine and bromodeoxyuridine or labeling by proliferating-cell nuclear antigen, which is implicated in the transition from G_1 to S phase.[3,4,20,24] However, these findings have been questioned as indicators of cell proliferation.[3,4] The detection of myocyte nuclei that are positive for thymidine and bromodeoxyuridine does not indicate whether DNA synthesis is coupled with nuclear hyperplasia, ploidy formation, or DNA repair. Furthermore, thymidine and bromodeoxyuridine cannot be injected into humans except in unusual circumstances.[25] Limitations apply to staining of proliferating-cell nuclear antigen in cell nuclei. Proliferating-cell nuclear antigen is a cofactor of DNA polymerase δ, which is implicated in DNA synthesis, cell-cycle progression, and DNA repair.[7] The last property may explain only in part the high level of expression of this protein in the nuclei of myocytes in terminally decompensated human hearts.[26] In fact, the reported values most likely overestimated the actual number of replicating my-

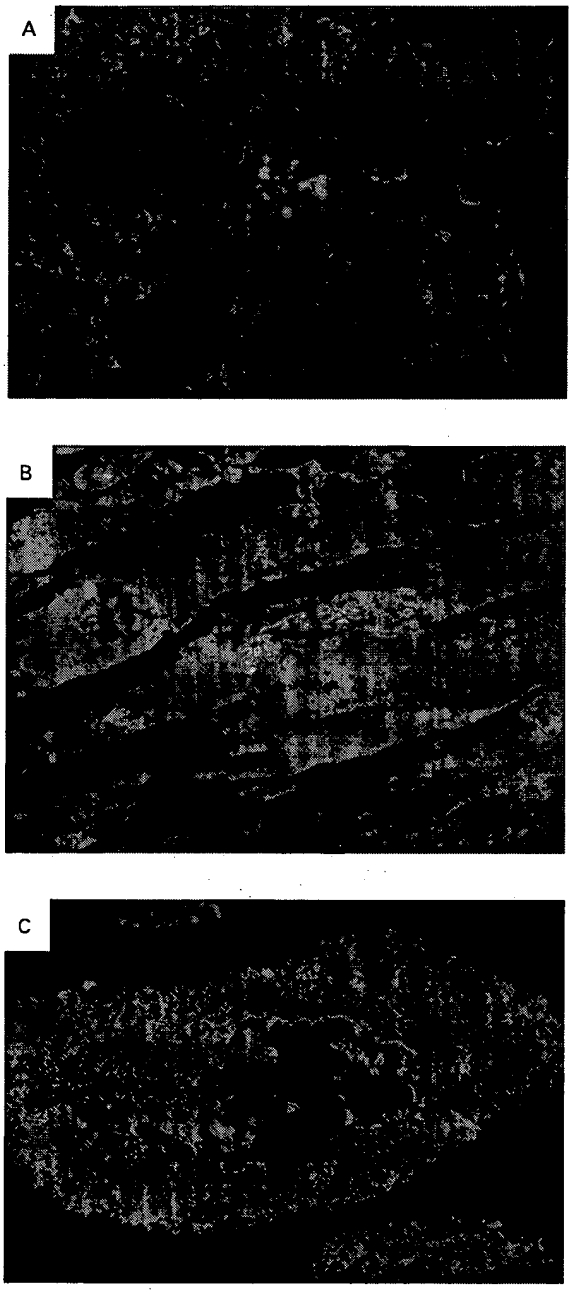


Figure 4. Mitotic Myocyte Nuclei in Infarcted Hearts.
Panels A, B, and C demonstrate the combination of labeling of myocyte cytoplasm by antibody against sarcomeric α-actin (red fluorescence) and staining of metaphase chromosomes by propidium iodide (green fluorescence). (Panels A and B, ×1200; Panel C, ×2000.)

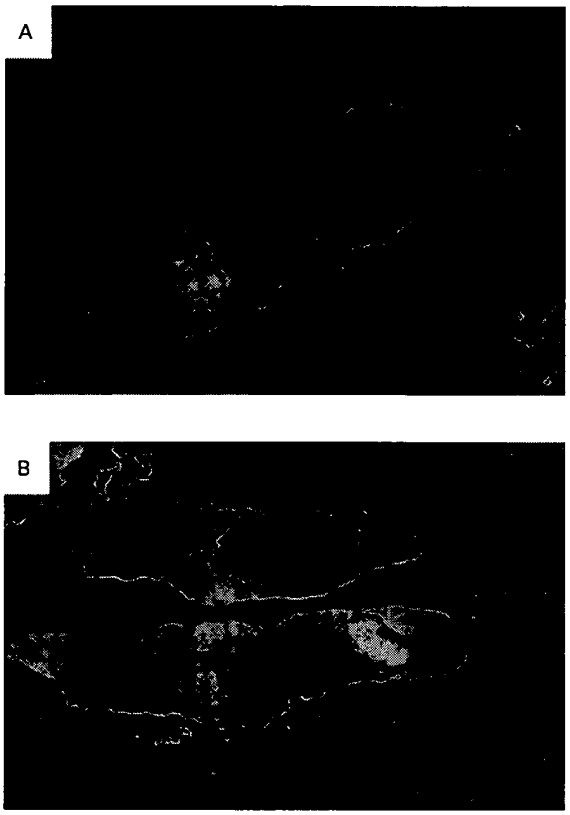


Figure 5. Myocyte Cytokinesis in Infarcted Hearts (×1500).
Cytokinesis is shown in Panels A and B by the combination of labeling of nuclei by propidium iodide (green fluorescence) and staining of myocyte cytoplasm by antibody against sarcomeric α-actin (red fluorescence). The divided nuclei mirror each other in the newly formed myocytes.

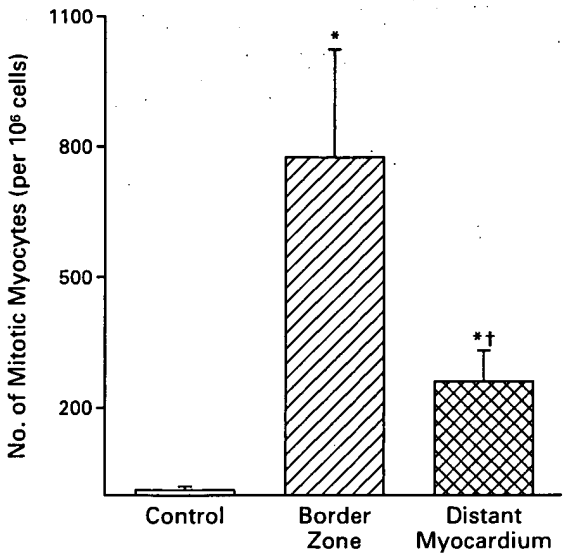


Figure 6. Effects of Infarction on the Mean (±SD) Number of Mitotic Myocytes.
The asterisks indicate P<0.001 for the comparison between the infarcted hearts and the control hearts; the dagger indicates P<0.001 for the comparison between the distant myocardium and the border zone in the infarcted hearts.

ocytes. We overcame these difficulties by using Ki-67 as a marker of cell proliferation. There is not a single example of a Ki-67–positive cell that cannot divide.[7-9,16,17] Biochemically, Ki-67 is an essential element of the outer dense fibrillar compartment of the nucleolus, where it acts as an efficiency factor in the rapid production of ribosomes for the increased metabolic requirements of dividing cells.[8] Structurally, Ki-67 is a molecule of 395 kd that contains a motif typical of several transcription factors.[27] Ki-67 has a preference for binding to adenine- and thymidine-rich sequences similar to the consensus site of *p53*.[9] This competition emphasizes the role of Ki-67 in cell replication.

The observation that mitotic indexes of nearly 800 and 300 myocyte nuclei per 10^6 cells characterize the acute myocardial response to infarction raises some crucial questions. The infarcted heart is frequently discussed as proof of the inability of myocytes to reenter the cell cycle and reconstitute muscle mass.[6,20] However, myocytes in the infarcted area die in a few hours, and ischemic damage occurs in the vascular and nonvascular components of the interstitium.[13] The formation of new myocardium in the infarcted region by myocyte growth alone is impossible. We found that mitotic activity occurred in myocytes in the border zone and the more distant myocardium, where tissue oxygenation was largely maintained.[28] The possibility that karyokinesis was not followed by myocyte cytokinesis is unlikely. Unlike the myocytes of rodents and dogs,[23,29,30] ventricular myocytes in humans are predominantly mononucleated.[31] Studies of dissociated myocytes from 72 normal hearts, 81 hearts with hypertrophy, and 95 hearts with ischemic cardiomyopathy, from subjects ranging from 26 to 93 years of age, found that mononucleated myocytes constituted 75 percent and binucleated cells 25 percent of the cell population. This proportion was not affected by disease, age, or sex.[31] However, this finding does not exclude the possibility that some binucleation of myocytes occurred after infarction.

Measurements of the proportion of myocytes in the cell cycle by labeling of Ki-67 and expression of the proportion of mitotic myocytes by means of the mitotic index indicate a consistent relation between these two markers of cell growth. The number of cycling myocytes is nearly 50 times as high as the number of mitotic myocytes in both normal and infarcted hearts.

Since mitosis is completed in about 30 minutes,[32] the duration of the myocyte cell cycle in vivo should be approximately 25 hours. The normal left ventricle contains 5.5×10^9 myocytes, and this value decreases to an average of 3.8×10^9 after myocardial infarction.[33] A mitotic index of 11 myocytes per 10^6 in the intact ventricle and 520 myocytes per 10^6 in the injured ventricle (775 myocytes per 10^6 in the border zone and 264 myocytes per 10^6 in the distant myocardium; mean, 520 myocytes per 10^6) implies that 60,500 myocytes are in mitosis in the normal left ventricle and 1,976,000 in the infarcted left ventricle. If the level of proliferation measured up to 12 days after coronary-artery occlusion persisted, the 1.7×10^9 myocytes lost as a result of infarction would be replaced in 18 days (myocytes per day, $1.98\times10^6\times48=95\times10^6$; myocytes per 18 days, $95\times10^6\times18=1.7\times10^9$). This calculation assumes that mitosis lasts 30 minutes (24 hours=48 half-hours) and that replicating myocytes divide only once during this period.

A relevant issue is the origin of cycling myocytes in normal and diseased hearts. These proliferating cells could derive from resident cardiomyocytes or from circulating stem cells that reach the spared myocardium after infarction. However, in the absence of stimulation by several cytokines, the number of circulating stem cells is very low.[34-36] Moreover, circulating stem cells move to the area of injury[36] without infiltrating the viable tissue.[37] Recently, we showed that bone marrow–derived stem cells, injected into the border of a myocardial infarct, homed to the infarcted zone and did not move into the remaining nonaffected portion of the ventricular wall.[37] Injury and large numbers of stem cells seem to be required for these cells' migration, multiplication, and differentiation into the cell lineages of the damaged heart or other organs.[37,38]

Although a cardiac stem cell has not yet been identified, such primitive undifferentiated cells may be present, and the dividing myocytes may be their progeny. This phenomenon occurs in the brain.[5,36] As in the damaged brain,[36] repair of the necrotic myocardium may involve interventions that promote the migration of endogenous, exogenous, or both types of stem cells to the infarcted region. Whether this therapeutic approach is superior to transplantation of myoblasts[39] or fetal cardiomyocytes[40] remains an important question. Hypertrophy and proliferation of myocytes do not prevent ventricular remodeling and the onset and evolution of cardiac failure after severe ischemic injury. Restoration of the infarcted myocardium, even in part, might interfere with the progression of the structural and functional alterations of the diseased heart,[37] thus delaying irreversible ventricular dysfunction.

In summary, our results challenge the dogma that the heart is a postmitotic organ. Myocyte proliferation may be a component of the growth reserve of the human heart; this mechanism could replace damaged myocardium. The presence of cell division in the nondiseased part of the heart suggests a continuous turnover of cells during the life span of the organism. The belief that myocardial infarction constitutes the most obvious demonstration of the incapacity of ventricular myocytes to replicate must be reconsidered.

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Heart 2001;86:481–482

Heart

Editorial

Can cardiomyocytes divide?

There is no doubt that research in the field of myocardial regeneration has a remarkably exciting future. This is reflected by a recent statement of the special emphasis panel at the National Heart, Lung, and Blood Institute in the USA, which was assigned to identify areas which should be in the focus of cardiovascular research within the next decades.[1] It says that we need to acquire " . . .fundamental understanding of stem cell biology, cardiogenic differentiation, and cell cycle control". However, there are certain limitations we face when we want to embark on this field of research:

(1) the availability of human tissue necessary to study cardiomyocyte division is limited and the heart consists of a heterogenous cell population;

(2) compared to the contractile protein apparatus there is only a low abundance of most cell cycle regulating factors;

(3) it may be necessary to convince people that counting cells alone may not give us the right answer about tissue regeneration.

If we wish to make progress in the field of myocardial regeneration in the nearer future, there are a number of issues that need to be addressed:

- which factors control cell cycle withdrawal in cardiomyocytes?
- what are their upstream regulators and what are their downstream targets?
- how do they interact with myogenic differentiation factors?
- how do they interact with hypertrophic and/or apoptotic signals in cardiomyocytes?

Cytokinesis

Cellular division is one of the key features and the basis of life in all metazoa. Therefore, any multicellular organ has to be endowed with a programme allowing cytokinesis. However, because of specific requirements some of these cells withdraw from the cell cycle during the perinatal period and become postmitotic. This is particularly true of cells comprising central organs such as the brain and the heart. For the latter, postnatal growth—which is essential to meet the increasing demand for work to support body growth—is achieved primarily by hypertrophy. This is probably because ongoing cellular division would render the heart mechanically unstable, and thus it would not be able to establish a functional circulatory homeostasis.

In contrast to skeletal muscle cells, where differentiation and cell division are mutually exclusive events, cardiac myocytes can contract and still divide; this is the case during fetal development, where the heart is responsible for providing oxygen rich blood to peripheral organs starting very early at embryogenesis, at a time where the heart itself is anything but fully differentiated. Thus, at an undifferentiated but already partially functional state cardiac cells can divide. This notion is in agreement with observations made in other organs which are known to retain a regenerative capacity throughout life. The liver, for example, has a regenerative capacity we have known about for a great many years, as reflected in the myth of Prometheus. Having stolen the secret of fire from the gods of the Olympus, Prometheus was condemned to having a portion of his liver eaten daily by an eagle. His liver regenerated overnight, thus providing the eagle with eternal food and Prometheus with eternal torture. However, not until recently have we realised that in order to divide even a liver cell has to retrodifferentiate.[2]

Several decades ago the German pathologist Linzbach postulated that the increase in the size of the failing heart could not be accounted for solely on the basis of hypertrophic growth.[3] However, he was unable to demonstrate cardiomyocyte mitosis. This may have been because of the limited technology available at that time. In modern times there are reports which claim to detect increased cardiomyocyte division in the failing[4] and infarcted[5] heart. Despite the availability of improved technology, these studies are flawed by invalid underlying hypotheses. Here, the authors calculate the number of mitotic cardiomyocytes in healthy and diseased failing myocardium on the basis of two incorrect assumptions: (1) the left ventricle contains 5.8×10^9 myocytes; and (2) the duration of mitosis lasts less than one hour and equals length of cell division. According to these assumptions the authors conclude that 0.001% of cardiomyocytes are in mitosis at a given time point, while there is an increase in mitosis in the failing myocardium by the order of one magnitude,[4] and by the order of nearly two magnitudes in the infarcted myocardium.[5]

To my knowledge there is no precedent for the assumption that any of our internal organs have a fixed number of cells as stated by these authors to be the case with the heart. Body size, sex, and age certainly contribute to a high variability among different individuals in regard to the number of cells in an organ like the heart. Secondly, the duration of mitosis may vary from cell to cell with organs exhibiting a high proliferation rate, where mitosis may take place within only 30 minutes, and organs with a rather slow proliferation rate, where mitosis may take place within 1–2 hours. Moreover, the extrapolation of the length of mitosis to the duration of cell division lacks any reasonable scientific basis. While the length of mitosis may vary by only a few hours between different cell types, the variation between the length of cell division may be several days as a result of the difference in the duration of G1 and G2 phase. If the analysis of the authors was correct, clinicians would encounter cardiac tumours much more frequently. However, primary myocardial tumours are rarely observed in adults, and if so are not of cardiomyocyte origin. Also, it is evident that functionally significant myocardial regeneration has not been documented in diseases and/or injuries that result in cardiomyocyte loss. So even if we accept the assumption that the adult heart is comprised of cardiomyocytes which have retained some degree of regenerative

growth, cardiomyocyte division appears to be such an unfrequent event that you do not have to be a cardiologist to know that after injury our heart is not able to rejuvenate.

Molecular mechanisms

Despite the fact, that this field of research has gained quite an impetus within the last few years, cell cycle control in cardiomyocytes remains enigmatic, if one ponders upon the potential molecular mechanisms accounting for the essentially irreversible cell cycle withdrawal of cardiomyocytes:

- the loss of growth factor receptors or signalling components—however, most growth regulating cascades persist into adulthood
- the loss of cell cycle proteins—however, most cell cycle proteins exist or can be reinduced in adult cardiomyocytes
- the elaboration of antiproliferative factors—however, animal models with disruption of candidate genes are not characterised by an overt cardiac phenotype
- the antiproliferative effect of myogenic determination factors—however, in cardiomyocytes differentiation and proliferation are not mutually exclusive events.

Does it look like this is going to be a hopeless endeavour? Certainly not! As outlined at the beginning of this article, mammalian cardiomyocytes are endowed with the full cell cycle programme, and although normal adult cardiomyocytes have down regulated levels of cell cycle factors, they retain the ability to re-express these factors during hypertrophic growth.[6] Thus, the critical question is not "Can cardiomyocytes divide?", rather it is "Why do cardiomyocytes not divide all the time?". With this modified question in mind we will progress much more quickly, because it implies that cardiomyocyte division is constantly suppressed through an active process. Thus, we may be able to induce cardiomyocyte division just by simply switching off this suppressive mechanism. This concept is fostered by the observation that cardiomyocyte cell cycle re-entry can easily be achieved by forced ectopic expression of cell cycle activators,[7] or by removing inhibitory cytoplasm from cardiomyocyte nuclei.[8]

How can we identify these switches which are responsible for the suppression of cardiomyocyte division? Again, there is a helping hand called programmed cell death or apoptosis. In general, cell death and cell cycle are tightly linked programmes which have to be kept in a fine tuned equilibrium.[9] A tight interplay between apoptotic signalling and cell cycle control has been described for a variety of different cell types, though there is still a lack of evidence in cardiomyocytes. According to this notion, the same factors should be responsible for the suppression of cell cycle and apoptosis. This is meaningful in a teleological sense, since a cell which is not allowed to divide should also be not allowed to die in order to preserve cellular homeostasis in a postmitotic organ such as the heart. Thus, studying anti-apoptotic signalling pathways most likely will lead to the identification of cell cycle checkpoints in cardiomyocytes. But beware—again the significance of apoptosis will not be recognised just by counting dead cells.

In conclusion, the initial question "Can cardiomyocytes divide?" can be answered with "Yes, they can!". However, at no time is the healthy or diseased human heart able to replace damaged tissue efficiently. The much more appropriate question "Why do cardiomyocytes not divide all the time?", I have to answer with "We do not know yet!". Nevertheless, searching for the molecular basis of cell cycle withdrawal and apoptosis in cardiomyocytes, rather than counting mitotic figures or dead cells, should provide us with critical information required to initiate interventions aimed at myocardial regeneration in the not too distant future.

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Progression From Compensated Hypertrophy to Failure in the Pressure-Overloaded Human Heart: Structural Deterioration and Compensatory Mechanisms

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Progression From Compensated Hypertrophy to Failure in the Pressure-Overloaded Human Heart

Structural Deterioration and Compensatory Mechanisms

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Background—The progression of compensated hypertrophy to heart failure (HF) is still debated. We investigated patients with isolated valvular aortic stenosis and differing degrees of left ventricular (LV) systolic dysfunction to test the hypothesis that structural remodeling, as well as cell death, contributes to the transition to HF.

Methods and Results—Structural alterations were studied in LV myectomies from 3 groups of patients (group 1: ejection fraction [EF] >50%, n=12; group 2: EF 30% to 50%, n=12; group 3: EF <30%, n=10) undergoing aortic valve replacement. Control patients were patients with mitral valve stenosis but normal LV (n=6). Myocyte hypertrophy was accompanied by increased nuclear DNA and Sc-35 (splicing factor) content. ACE and TGF-β_1 were upregulated correlating with fibrosis, which increased 2.3-, 2.2-, and 3.2-fold over control in the 3 groups. Myocyte degeneration increased 10, 22, and 32 times over control. A significant correlation exists between EF and myocyte degeneration or fibrosis. Ubiquitin-related autophagic cell death was 0.5‰ in control and group 1, 1.05 in group 2, and 6.05‰ in group 3. Death by oncosis was 0‰ in control, 3‰ in group 1, and increased to 5‰ (groups 2 and 3). Apoptosis was not detectable in control and group 3, but it was present at 0.02‰ in group 1 and 0.01‰ in group 2. Cardiomyocyte mitosis was never observed.

Conclusions—These structure-function correlations confirm the hypothesis that transition to HF occurs by fibrosis and myocyte degeneration partially compensated by hypertrophy involving DNA synthesis and transcription. Cell loss, mainly by autophagy and oncosis, contributes significantly to the progression of LV systolic dysfunction. (***Circulation.* 2003;107:984-991.)**

Key Words: hypertrophy ■ heart failure ■ structure ■ remodeling ■ hemodynamics

The structural basis of the progression from well-compensated hypertrophy caused by mechanical overload to heart failure (HF) is still largely unknown. It is evident that cardiac remodeling, defined as "genome expression, molecular, cellular, and interstitial changes that are manifested clinically as changes in size, shape and function of the heart after injury" occurs in the chronically pressure-overloaded heart.[1] However, the correlation between morphological alterations and clinical data during the different phases of transition to HF has not yet been described in the human heart.

Krayenbühl and colleagues[2] in 1989 described fibrosis and myocyte enlargement in patients with aortic stenosis (AS) with normal ejection fraction (EF) but elevated left ventricular end-diastolic pressure (LVEDP). They studied cardiac functional recovery after aortic valve replacement (AVR) but not the progression to HF. In end-stage human HF, we reported fibrosis as well as impairment of the myocyte ultrastructure defined as degeneration.[3] Anversa et al[4] and others (review by Elsässer et al[5]) emphasized cell death, mainly by apoptosis, as one of the key events for the occurrence of failure.

Fibrosis and myocyte damage appear to be the decisive morphological alterations in the remodeling process.[6,7] The most important regulators of fibrosis are the members of the renin-angiotensin and aldosterone system.[8,9] In addition, TGF-β_1 is a potent stimulator of fibrosis, as are growth factors and endocrine hormones such as norepinephrine (reviewed by Hein and Schaper[10]). Recent studies in small rodents have shown that the development of fibrosis is prevented in the absence of TGF-β_1, indicating the crucial fibrosis-promoting role of this cytokine.[11,12]

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TABLE 1. Clinical Preoperative Data

	Control	Group 1	Group 2	Group 3
Age, y	68±6	70±6	67±12	71±7
Men/women	4/2	5/7	5/7	6/4
EF, %	61±8	59±8	41±5†	24±5†
LVEDP, mm Hg	8±1	15±5	18±6†	24±5†
PCWP, mm Hg	21±2*	13±6	12±5	20±9
PAPmean, mm Hg	28±4*	19±4‡	18±5	30±8
ΔPmean, mm Hg	11±3§	62±17	58±14	47±17
Valve orifice, cm^2	0.9±0.5§	0.6±0.2	0.7±0.1	0.5±0.2
LVSP, mm Hg	130±17	191±25†	180±15†	156±15
LVEDD, mm	50±2	45±7‡	48±6	55±6
LVESD, mm	32±3	29±8‡	34±6	41±11
LV septum WT, mm	11±1	15±2†	14±2†	13±3
LV relative WT	0.42±0.01	0.63±0.14	0.5±0.18	0.45±0.1
LV mass/m^2	104±14	137±26	131±57	153±35†
Atrial fibrillation‖	5	1	2	6
LV decompensation‖	0	0	2	7
Diuretics‖	4	2	7	9
Digitalis‖	5	1	3	6
ACE inhibitors‖	2	2	2	1
β-Blockers‖	3	3	4	3

All patients with regurgitation of aortic/mitral valve ≤1 degree.

Etiology: n=1 bicuspid, n=11 degeneration, n=22 postendocarditis.

*PCWP and PAP pressure in control subjects are elevated because of MS (real control values <10 and <18 mm Hg, respectively).

†$P<0.05$ compared with control; ‡$P<0.05$, group 3 vs group 1; all others not significantly different.

§Mitral valve orifice and pressure gradient in control subjects.

‖No. of patients.

Earlier, we described myocyte degeneration and loss in the transition to end-stage HF.[3] In the present study, we investigated patients with AS in different hemodynamic situations ranging from compensatory hypertrophy with intact systolic function to symptomatic HF with depressed systolic function. We studied the relation between clinical LV parameters and morphological alterations including fibrosis, myocyte degeneration, and cell death. To test the assumption that compensatory mechanisms may be active, we determined the DNA content and the presence of the splicing factor Sc-35.[13] The

TABLE 2. Morphometric Data

	Control	Group 1	Group 2	Group 3
Myocyte No./mm^2	380±20	366±45	290±21*	216±32*
Cross-sectional area, μm^2	424±37	561±91	658±84*	593±64*
Myocytes <300 μm^2, %	2.5±2	10.5±3*	6.2±4	17±5*
Longitudinal cell area, μm^2	1397±76	2268±203	2488±481*	2397±559*
Nuclear area, μm^2	71±7	80 ± 6	87±5	103±5*
Nuclear/myocyte area	0.05	0.04	0.04	0.04
Ki-67, n/mm^2	0.11±0.09	0.25±0.09	0.55±0.23	0.3±0.13
CD3, n/mm^2	1.3±1.6	3.8±3.2	3.3±2.7	4.3±3.3
CD68, n/mm^2	32±4	63±18*	56±5	59±9*
CD45, n/mm^2	4.8±0.8	15.2±2.3*	10±1.2*	12±2.7*
Ubiquitin positive, ‰	0.5±0.01	0.5±0.03	1±0.4*	6±0.5*
C9 positive, ‰	0	3±0.5*	5±0.8*	4±0.3*
Apoptosis, ‰	0	0.02±0.002	0.01±0.003	0
Myocyte degeneration, ‰	0.5±0.4	5.1±4.1	11.4±5.3*	15.9±8.7*

*$P<0.05$ vs control, †$P<0.05$ vs group 1.

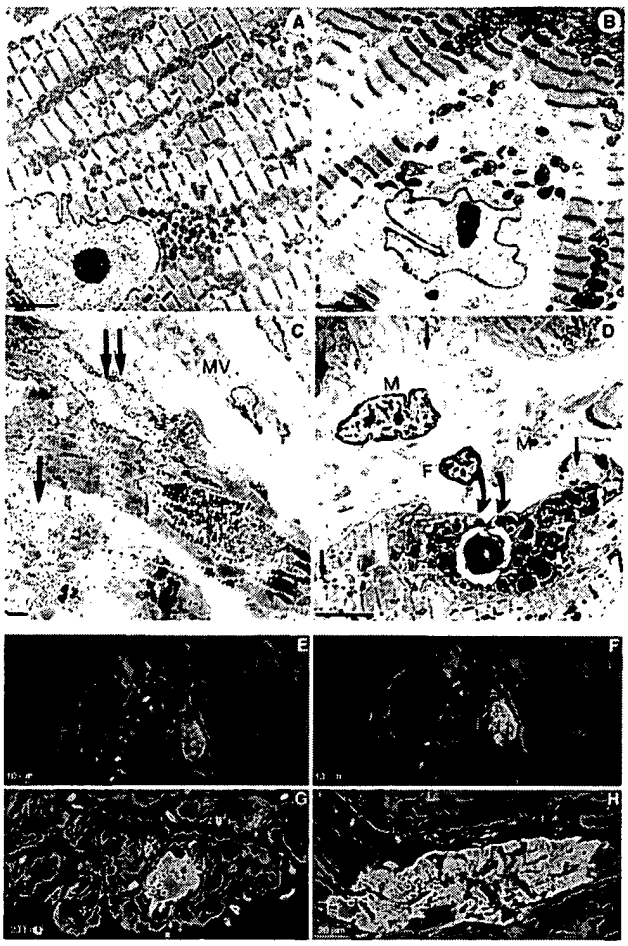

Figure 1. Degeneration and cell death. A through D, Electron microscopy; E through H, CLSM. A, Normal; B, slight degeneration with perinuclear cytoplasm; C, loss of sarcomeres ranging from slight (arrow) to severe (double arrow); MV, microvessel; D, one atrophied myocyte (curved arrow) contains autophagic vacuoles; the others lack sarcomeres (single arrows); F, fibroblasts; M, macrophages. All bars are 5 μm. E and F, Apoptosis. E, Myocyte (red) nucleus is blue with TOTO and green by TUNEL in F. G, Ubiquitin labeling (green) in a myocyte (red) nuclei are blue. Note absence of nucleus in affected cell. H, Labeling of C9 (green) in a myocyte. Colors are as in G.

final goal of this work was 2-fold: (1) To define the mode of transition of compensated hypertrophy to HF in the pressure-overloaded human heart with emphasis on the role of cell death: autophagy associated with the ubiquitin-proteasomal pathway,[13,14] oncosis (necrosis is cellular breakdown after cell death has occurred[15]), and apoptosis,[16] and (2) to determine the correlation between preoperative/postoperative clinical data and morphological findings, which might determine the potential for complete postoperative recovery.

Methods

Patients

Thirty-four patients with isolated AS underwent clinical evaluation (Table 1) and were subdivided into 3 different groups on the basis of EF determined by quantitative echocardiography at the time of admission: group 1, EF >50% (n=12); group 2, EF 50% to 30% (n=12); group 3, EF <30% (n=10). All patients underwent surgical AVR and postoperative examination (identical n per group). Six patients with mitral stenosis (MS) with normal EF served as control subjects. The institutional ethics committee approved the study, and all patients gave informed consent.

Tissue Sampling

During open heart surgery, myectomy samples weighing ≈30 to 80 mg were removed from the LV septum, immediately frozen in liquid nitrogen, and stored at −80°C. In MS, samples from papillary muscles were obtained. In addition, small samples were fixed in buffered glutaraldehyde for electron microscopy.

Electron Microscopy

The samples were embedded in Epon, following a standard protocol. Ultrathin sections were double-stained with uranyl acetate and lead citrate before examination in a Philips CM 10. Myocyte degeneration, defined as loss of contractile elements and disorganization of ultrastructural organelles, was evaluated quantitatively by two observers blinded to the patient group.

Immunolabeling and Confocal Microscopy

Cryosections 5 μm thick were air-dried and fixed either with paraformaldehyde or acetone. Primary antibodies were fibronectin (rabbit polyclonal, ICN), TGF-β_1, ACE (Chemicon), CD3 (lymphocytes), CD31 (endothelial marker, PECAM), CD45 (panleukocytes), CD68 (macrophages), and Ki-67 (DNA synthesis) (all Dako), vinculin, sarcomeric α-actinin, and Sc-35 splicing factor (all Sigma), ubiquitin for autophagic cell death, (Zymed Laboratories), complement 9 (C9, Serotec) for oncosis, and the TUNEL method for apoptosis (Roche). The specificity of all antibodies was verified by omission of primary antibodies.

The secondary detection system was biotinylated anti-mouse or anti-rabbit IgG (Biotrend) either directly conjugated with Cy-2 or Cy-3 or unconjugated followed by fluoroisothiocyanate (FITC)-linked streptavidin (Amersham). Myocyte identification was done with TRITC-labeled phalloidin (Sigma). Nuclei were stained with TOTO-3 (Molecular Probes). Picture acquisition was performed with a Leica confocal microscope (CLSM); data were transferred to a Silicon Graphics workstation for further processing and recording (Bitplane software).

Morphometry

Myocyte degeneration was evaluated in the confocal microscope from α-actinin–stained sections at a magnification of ×400. The total myocyte number was determined and degeneration calculated as percentage. Myocyte cross-sectional and longitudinal cell areas were determined directly in the confocal microscope from at least 120 myocytes per section by delineating vinculin-stained myocytes.

Fibrosis and Numerical Densities

Fibrosis was quantified from fibronectin-stained sections from 5 different fields of vision randomly chosen and expressed as percentage of total myocardium. Capillary density was determined from 5 different fields of PECAM-stained sections and calculated (as n/mm^2). The same was done for ACE-positive microvessels as well as for CD3, CD45, CD68, and Ki-67. All densities were determined at a microscopic magnification of ×250.

Cell Death

Numbers of ubiquitin-, C9-, and TUNEL-positive cells were obtained from the entire section. Section size and number of myocytes per mm^2 were determined, and percentages of positive myocytes were calculated. The total number of myocytes evaluated per patient varied between 2748 and 5976.

Fluorescence Intensity by Confocal Microscopy

The immunolabeling procedures for TGF-β_1 were carried out under identical conditions, including the microscopic magnification of ×400. Confocal settings were kept constant, and quantification of TGF-β_1 was performed by measurements of fluorescence intensity

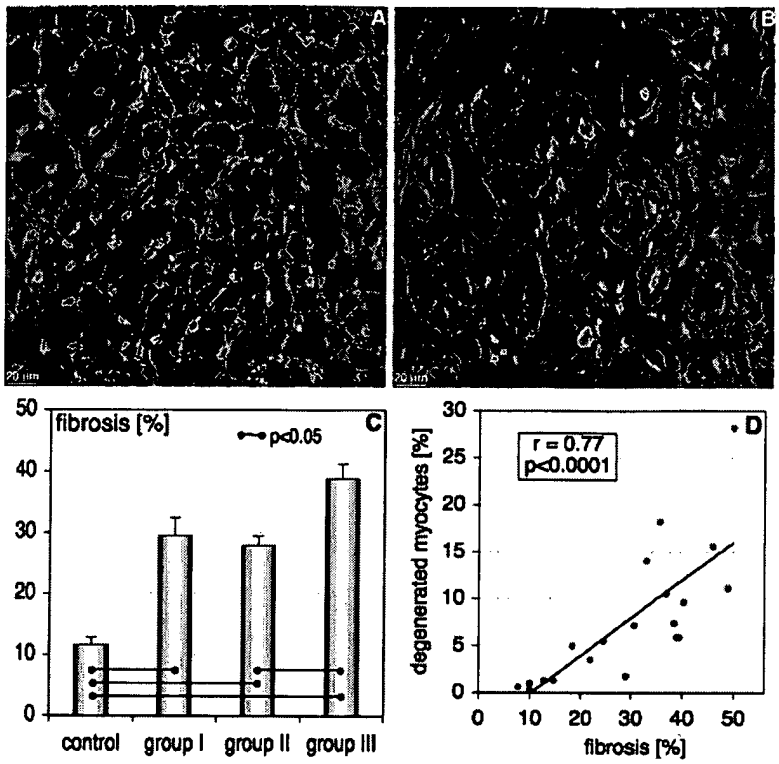

Figure 2. Fibronectin staining (red, nuclei blue). A, Normal myocardium shows fine septa between unstained myocytes; B, severe fibrosis with few myocytes; C, fibrosis is already severe in group 1; D, significant correlation exists between fibrosis and myocyte degeneration.

by using a range of 0 to 255 values. Arbitrary units were calculated per unit surface area (AU/mm^2).

FEULGEN Staining and Quantitative Evaluation

Nuclear DNA was stained by the FEULGEN method. All sections were stained simultaneously under exactly the same protocol. Quantitative evaluation of the nuclear DNA content (total amount times nuclear area) and concentration (DNA/nuclear area) was done with the CLSM at a magnification of ×400. At least 400 nuclei per sample were measured. The same procedure was followed for Sc-35. Nuclear area was automatically obtained from measurements of FEULGEN staining.

Statistics

All data are presented as mean±SEM. Differences by unpaired *t* test, ANOVA, Bonferroni, or Kruskal-Wallis were considered significant when $P<0.05$.

Results

Myocyte Hypertrophy and Degeneration

Myocytes showed hypertrophy. The cross-sectional and longitudinal cell areas were increased in all groups compared with the control (MS) group (Table 2). Interestingly, the number of atrophied myocytes, defined as cells with a cross-sectional area <300 μm^2, was rather large (Table 2).

Subcellular changes in myocytes by electron and confocal microscopy were reduction of sarcomeres, occurrence of myelin figures and autophagic vacuoles, numerous polysomes, and nuclei of bizarre shape. The percentage of myocyte degeneration/total number of myocytes was slightly increased in group 1 and significantly elevated in groups 2 and 3 (Figure 1 and Table 2).

Ubiquitin-related autophagic cell death and oncosis increased with depressed EF (Figure 1 and Table 2). In group 3, with severe LV dysfunction, the prevalence of myocytes undergoing autophagic and oncotic death was ≈5‰ and 4‰, respectively, whereas classic apoptosis was detected in <1 per 10 000 myocytes.

Fibrosis

Fibrosis was already significantly increased in group 1 as compared with control; it remained unaltered in group 2 and was elevated to almost 40% in group 3 (Figure 2, A through C). A significant correlation exists between the degree of fibrosis and myocyte degeneration (Figure 2D).

Hemodynamic Status

Significant pressure overload with increased LVSP was present in all patients as the result of severe AS, with orifice surface ranging between 0.96 to 0.22 cm^2. The earliest changes caused by chronic pressure overload were an elevation of LVEDP and wall thickness. With decreasing EF, a further increase of LVEDP and as sign of pulmonary congestion an elevation of pulmonary artery pressure was found. Maximal levels of LVSP, ΔP, and relative wall thickness were found in group 1 and decreased in group 3. LV mass tended to be higher in the patients with AS compared with control (MS) patients. Atrial fibrillation occurred more frequently in group 3 than in the other groups.

There was a good correlation between percentage of degenerated myocytes and EF as well as correlations between percentage fibrosis and both EF and LVEDP (Figure 3, A through C). Postoperative evaluation at 2.6±1.4 years after AVR showed that EF was within normal range in group 1 and 2 patients; in contrast, group 3 patients showed continued LV systolic dysfunction associated with persistent depression of

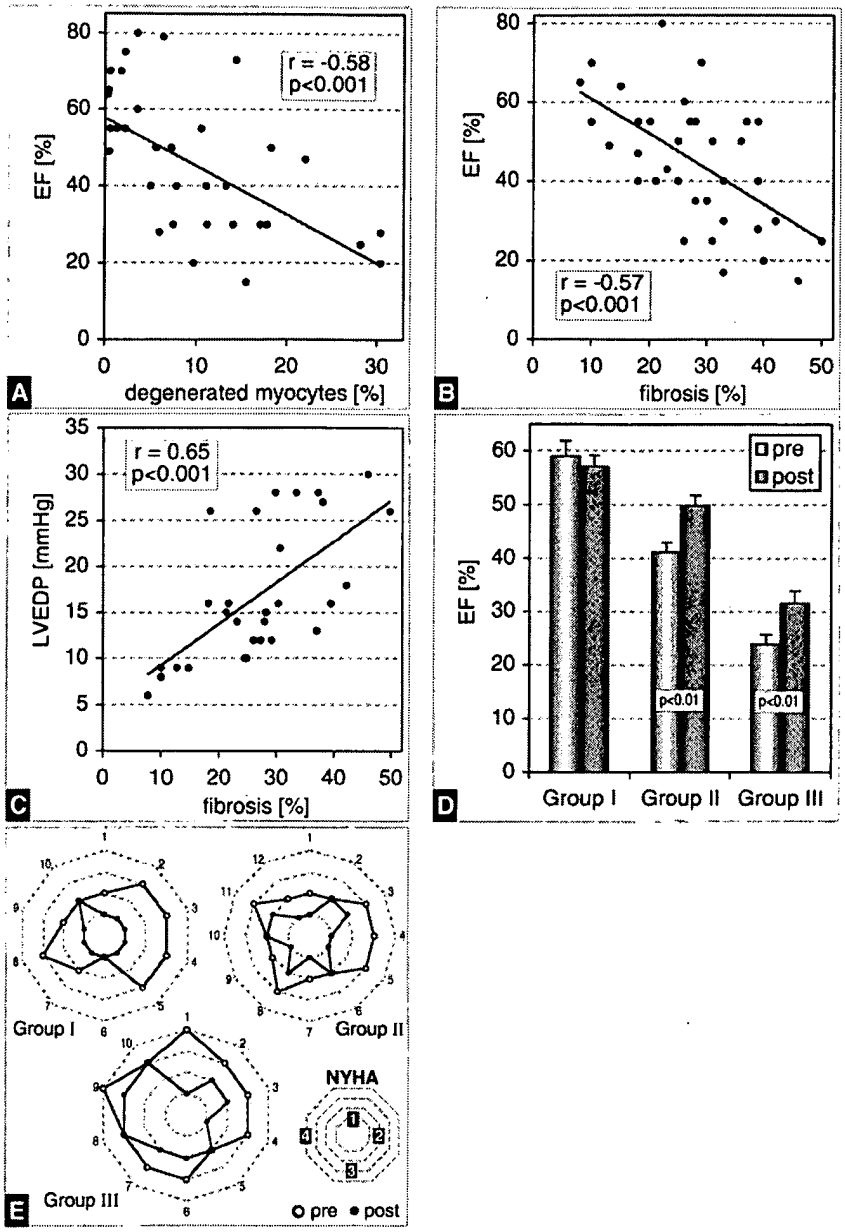

Figure 3. A through D, Good correlation exists between clinical data, degeneration, and fibrosis. D through E, Improvement after surgery. D, Recovery of EF; E, recovery of NYHA class. D and E document that adaptation to pressure overload is present until group 2 but that group 3 has reached a critical hemodynamic state with exhaustion of adaptational processes and incomplete recovery.

New York Heart Association functional class (Figure 3, D and E).

Inflammation Markers

There was a 3-fold increase in leukocytes (CD45) but only a slight increase in the number of lymphocytes (CD3) and macrophages (CD68) situated in the perivascular and interstitial space (Table 2).

ACE, TGF-β_1, and Capillary Density

The number of CD31-positive microvessels was reduced (Figure 4C). ACE was present in the endothelium of microvessels (Figure 4A) and increased in all groups when expressed as a percentage of capillary density (Figure 4C). TGF-β_1, localized in fibroblasts and macrophages (Figure 4B), was elevated in all groups (Figure 4D).

Nuclear DNA and Sc-35

The content (AU times nuclear area) of DNA and SC-35 was increased in all groups, but the concentration (AU/nuclear area) remained unchanged (Figure 5, A through D). However, since the ratio of nucleus/cell area was decreased (Table 2), the elevation of content is insufficient for the enlarged myocytes. The number of Ki-67–positive myocytes and of binucleated myocytes was unchanged in all groups (Table 2).

Discussion

In this study, we present evidence that a close correlation exists between cardiac function and myocardial morphology in patients with AS. With worsening of fibrosis and myocyte degeneration, LVEDP increases and later EF decreases. This suggests that a structure-function relation leading to HF is present in human pressure overload. This correlation is also

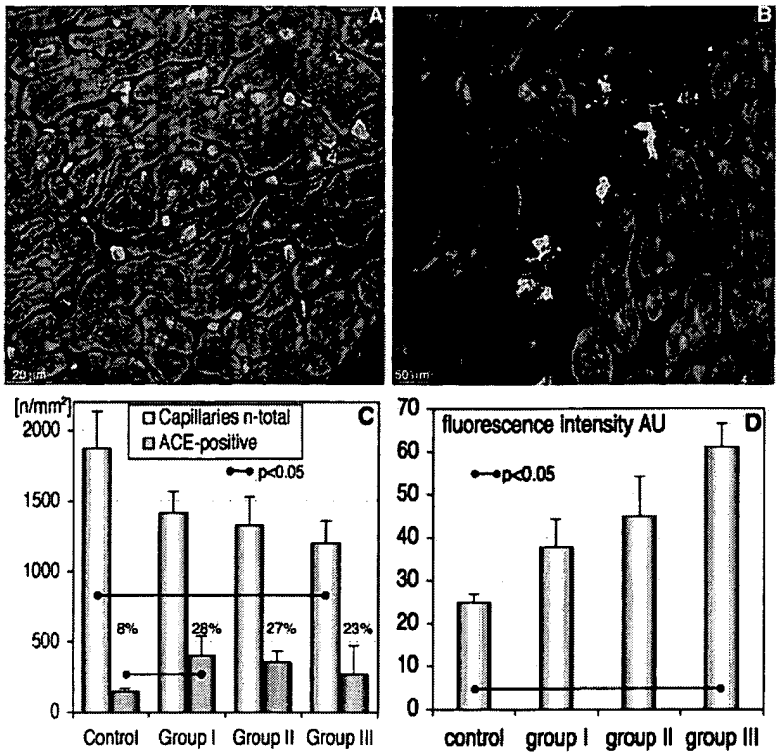

Figure 4. ACE and TGF-β_1 (green, myocytes red, nuclei blue). A, ACE is localized in numerous microvessels. B, TGF-β_1 is present in fibroblasts and extracellular matrix. C, Number of ACE-positive microvessels is significantly elevated in group 1 and remains almost constant when expressed as percentage of total number of capillaries. D, TGF-β_1 increases in later stages of hypertrophy.

reflected in the degree of postoperative recovery, which was incomplete in patients group 3. This should be taken into account for the decision of surgical intervention.

Fibrosis is an early morphological alteration in patients with AS. It is a major determinant of diastolic dysfunction and systolic pumping capacity,[17] and it is one of the structural substrates for arrhythmogenicity, thus playing a major role for sudden death and the progression of HF.[18] This is in agreement with Krayenbühl et al,[2] who performed serial observations in the same patients and found a partial regression of severe fibrosis 6 to 7 years after AVR, which is unlikely to occur in the patients of group 3 of this study.

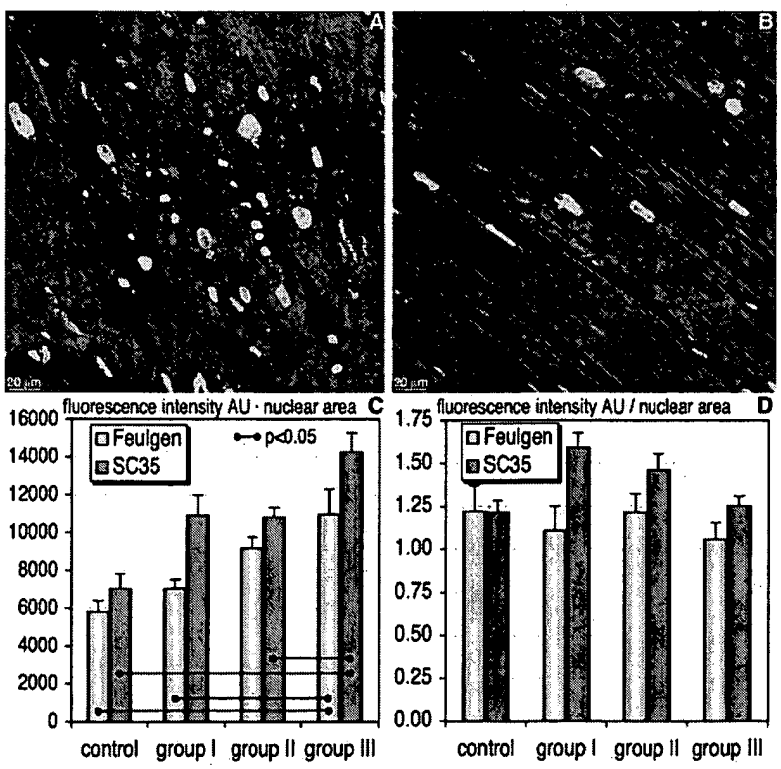

Figure 5. DNA and Sc-35 labeling (myocytes red, nuclei green for either DNA [A] or Sc-35 [B]). Yellow dots are lipofuscin granules. A, Typical staining pattern for DNA in nuclei. B, Sc-35 localization. C, Nuclear content of Sc-35 and DNA. D, Nuclear concentration of Sc-35 and DNA.

Inflammation was of low grade and reflected in the increase in leukocytes and macrophages observed. These may be responsible for the increase in cytokine production (TGF-β_1, TNF-α, interleukin family) that accounts not only for the inflammatory response but also for the formation of reactive fibrosis.

Previous studies of myocardium from patients with AS have shown that ACE and TGF-β_1 were upregulated on the mRNA and protein level.[19] Because TGF-β_1 was upregulated in group 1, we suggest that TGF-β_1 is one of the major determinants of fibrosis progression, thus confirming studies by others (reviewed in Reference 20).[20] ACE was elevated in group 1. De novo synthesized angiotensin II locally released by the action of ACE regulates TGF-β_1 production, and it is this fibrogenic cytokine that regulates the collagen turnover of fibroblasts.[9] The present findings corroborate the hypothesis that angiotensin release by the action of ACE is stimulated by a paracrine mechanism involving TGF-β_1 as a mediator.[21]

Myocyte Degeneration

The degree of degenerative injury of cardiomyocytes increased with the development of HF. The term degeneration was chosen to emphasize involvement of all cellular organelles in a chronic and most probably slow process of degradation that finally results in cellular atrophy, myocyte death, and replacement fibrosis. The severity of changes exceeded those typically observed in myocardium from patients with dilated cardiomyopathy.[3] The significant correlation between myocyte degeneration with fibrosis and with EF suggests a mutual influence of cardiac structure and function.

DNA Replication, Transcription, and Lack of Mitosis

Compensatory mechanisms were, however, observed as well. The increase in SC-35, an RNA splicing factor, indicates ongoing transcription in all groups. Sc-35 belongs to the group of non-snRNP factors and is required for the first step of splicing and spliceosome assembly.[22] We would like to postulate that the presence of Sc-35 indicates that the cardiomyocytes, even when damaged, are viable and capable of transcription and translation.

This assumption is reinforced by the DNA data presented in this report, which confirm earlier experimental studies.[23,24] The ability of the human myocyte to increase its DNA content avoids "dilution" of the DNA in the enlarged cells and permits DNA repair, thus allowing for a significant hypertrophic response. However, since the nucleus/cell volume ratio is disturbed, the amount of DNA is not sufficient to sustain the transcriptional levels required by the enlarged cell volume and cellular exhaustion will be the consequence. Data published by other groups support the notion that DNA synthesis takes place in the adult mammalian heart,[24] and this is strongly suggested by our own data as well.

The cell-cycle-associated nuclear nonhistone Ki-67 protein is expressed in all active phases of the cell cycle but not in quiescent G_0 cells.[25] It is more abundant in DNA synthesis and mitosis but it might also be observed when DNA synthesis is inhibited.[26] The number of binucleated myocytes did not increase with hypertrophy, and therefore we assume that the presence of Ki-67–positive myocytes reflects DNA replication for maintenance of the DNA content/myocyte volume ratio and for DNA repair but not mitotic nuclear division,[26] confirming results by other groups.[27,28]

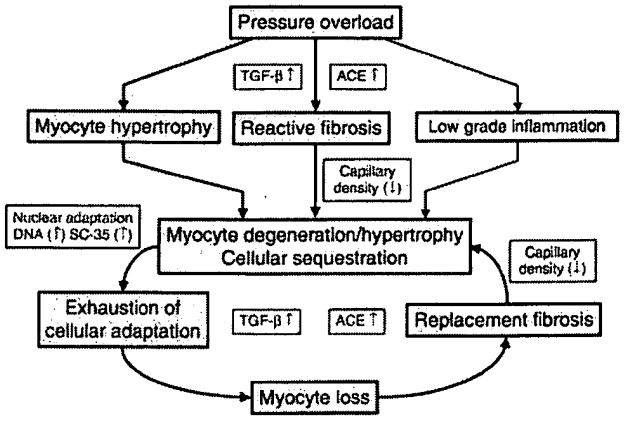

Figure 6. Schematic illustration of continuous remodeling in pressure-overload hypertrophy.

Mitotic figures indicative of a putative regeneration process were never observed. This contrasts with another study[29] but confirms earlier work in human and animal tissue.[30] Since cardiomyocyte mitosis was completely absent, the present data implicate that myocyte degeneration will lead to final cell loss.

Ubiquitin-related autophagic cell death[13,14] and oncosis[31] appear to be more important than apoptosis, which occurred at an extremely low rate. Our data suggest that ubiquitin binds contractile or membrane proteins destined for degradation but that because of proteasomal insufficiency, the complexes are accumulated and might cause nuclear fragmentation (unpublished). Myocytes therefore exhibit large areas with loss of cross-striated sarcomeres. Cells will disintegrate and will be replaced by fibrosis. Narula's concept[16] that activated caspase causes myocyte protein degradation without nuclear DNA fragmentation might be important in that regard and will be pursued in further studies. Single-cell oncosis appears to originate from reduced coronary flow reserve and diffusion disturbances caused by fibrosis and the reduction of capillary density.

On the basis of the data presented here, we depict in Figure 6 a model of the major adaptation to pressure overload. It may be concluded that reduction of cardiac function occurs when mechanisms such as DNA repair and synthesis as well as Sc-35 expression are partially or totally exhausted, when myocyte death occurs and fibrosis will have reached a certain degree. These changes will affect an increasing number of myocytes and HF will finally occur. A self-perpetuating process of myocyte degeneration, cell death, and replacement fibrosis will be maintained, even when excessive afterload will return to normal after AVR. This chronic cycle will lead to further impairment of LV function and poor prognosis.

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Cardiac Myocyte Cell Cycle Control in Development, Disease, and Regeneration

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Ahuja P, Sdek P, MacLellan WR. Cardiac Myocyte Cell Cycle Control in Development, Disease, and Regeneration. *Physiol Rev* 87: 521–544, 2007; doi:10.1152/physrev.00032.2006.—Cardiac myocytes rapidly proliferate during fetal life but exit the cell cycle soon after birth in mammals. Although the extent to which adult cardiac myocytes are capable of cell cycle reentry is controversial and species-specific differences may exist, it appears that for the vast majority of adult cardiac myocytes the predominant form of growth postnatally is an increase in cell size (hypertrophy) not number. Unfortunately, this limits the ability of the heart to restore function after any significant injury. Interest in novel regenerative therapies has led to the accumulation of much information on the mechanisms that regulate the rapid proliferation of cardiac myocytes in utero, their cell cycle exit in the perinatal period, and the permanent arrest (terminal differentiation) in adult myocytes. The recent identification of cardiac progenitor cells capable of giving rise to cardiac myocyte-like cells has challenged the dogma that the heart is a terminally differentiated organ and opened new prospects for cardiac regeneration. In this review, we summarize the current understanding of cardiomyocyte cell cycle control in normal development and disease. In addition, we also discuss the potential usefulness of cardiomyocyte self-renewal as well as feasibility of therapeutic manipulation of the cardiac myocyte cell cycle for cardiac regeneration.

I. INTRODUCTION

Adult cardiac myocytes represent a highly specialized and structured cell type; therefore, it is not surprising that complex and often overlapping systems have evolved to regulate cardiomyocyte growth. Cardiac myocytes rapidly proliferate during fetal life, but in the perinatal period, proliferation ceases and myocytes undergo an additional round of DNA synthesis and nuclear mitosis without cytokinesis (acytokinetic mitosis) that leaves the majority of adult cardiac myocytes binucleated in most species (131, 132). Typically, adult cardiac myocytes do

not reenter the cell cycle when exposed to growth signals, and further increases in cardiac mass are achieved through an increase in cell size or hypertrophy. Thus cardiac myocytes display three developmentally determined forms of cell cycle control and growth, namely, proliferation, binucleation, and hypertrophy (see Fig. 2).

Many mammalian tissues respond to injury by activating committed progenitor or stem cells or through proliferation of differentiated cells capable of reentering the cell cycle, although both have been felt to be quite limited in the heart until recently (21). Several groups have described the presence of a progenitor or stem cell that can differentiate into cardiac myocytes (56), but there is still much debate and confusion in the field regarding the exact identity of this cell and its potential for cardiac myocyte differentiation and myocardial repair (8, 195). Nonetheless, most investigators would agree that adult cardiomyocytes have very limited potential for self-renewal, which is inadequate to repair the heart after significant injury. The failure of the adult mammalian myocardium to reactivate the cell cycle has been postulated to be a primary limiting factor in restoring function to the damaged heart. Thus, although limited induction of DNA synthesis in the heart as a response to stress or other factors has been described (29, 70), there is little evidence for cytoplasmic division or cytokinesis in mammals. In contrast, cardiomyocytes from lower vertebrates are capable of dividing postnatally (158, 167). The mechanisms underlying these species differences are unknown.

One major limitation in the field has been the lack of adequate models or techniques in mammalian systems to truly mechanistically analyze cardiac myocyte proliferation and differentiate cell cycle reentry from its downstream consequences including increased ploidy, nuclear mitosis, cytokinesis, and apoptosis. Currently no clonal cells lines exist that reproduce the normal developmental pattern of cardiac myocyte cell growth. Thus investigators have been forced to utilize primary cell cultures despite the knowledge that there are many physiological and molecular differences between fetal or neonatal myocytes and postmitotic adult myocytes. The fact that neonatal myocytes retain some proliferative potential and express endogenous cell cycle activators makes them a less than ideal model for studies designed to understand cell cycle regulation in the adult heart. Likewise, difficulties associated with culturing adult myocytes for extended periods and their tendency to dedifferentiate and regain proliferative potential also limit their usefulness for in vitro cell cycle analyses (39). Thus, although not without their own shortcomings, we have given special emphasis to in vivo studies using transgenics and gene-targeting technologies. However, there are certain caveats that should be kept in mind when considering the data generated from genetically altered mouse models. Many of these transgenics have utilized constitutive, albeit cardiac-restricted, expression of genes implicated in cell cycle proliferation. This typically results in expression at developmental time points where the myocyte retains proliferative potential. We and others (50) have shown in species as diverse as *Drosophila* and mouse that the effects of overexpression of some cell cycle proteins is dependent on whether it occurs in a cell retaining proliferative capacity versus a cell that is postmitotic. Furthermore, as we outline below, cell cycle reentry can have several outcomes, only one of which is cytokinesis, and even the documentation of mitotic figures does not necessarily imply proliferation given that cardiac myocytes are well-known to be multinucleated. Despite these confounding factors, much useful information has been gained from generation and characterization of these genetically altered mice.

This review focuses on the progress that has been made in the field of cardiac growth control in mammals with an emphasis on several broad questions. What are the signals or molecules driving the rapid cardiac myocyte proliferation in utero, and what developmental mechanisms later block the capacity for proliferative growth? What potential does the heart have for regeneration? Do the same factors that regulate hyperplastic growth also mediate hypertrophic growth in adult postmitotic myocytes? The answers to these questions have important implications not only for understanding cardiac development and disease but will also serve as a foundation towards manipulating cardiac growth for therapeutic benefit in the future.

II. DEVELOPMENTAL CONTROL OF CARDIAC MYOCYTE PROLIFERATION

A. Temporal Pattern of Cardiac Growth in Mammals

Cardiac myocytes rapidly proliferate during fetal life but lose their ability to proliferate soon after birth; however, before terminal withdrawal from the cell cycle, cardiomyocytes undergo a final round of incomplete cell division, during which karyokinesis gets uncoupled from cytokinesis, resulting in binucleated cardiomyocytes. Analysis of cardiac myocyte proliferation during mouse development determined that cardiac myocyte DNA synthesis occurs in two distinct phases (213). The first occurs during fetal life, peaking at the earliest time points measured, where labeling indexes of 33% were observed in ventricles of embryonic day 12 fetuses. During this phase, karyokinesis and cytokinesis were matched, resulting in cardiac myocyte proliferation. The second phase occurred early in the neonatal period, peaking at day 4–6 postnatally. In contrast, in this phase, karyokinesis occurred in the absence of cytokinesis, resulting in binucle-

ation of the ventricular myocytes. This process of nuclear division in the absence of cellular division is a specific form of endoreduplication known as acytokinetic mitosis. Interestingly, it was shown that these cardiomyocytes still assemble an actomyosin contractile ring in culture, but abscission no longer takes place (131). In mice and rats, the accumulation of binucleated cardiomyocytes starts around day 4, and by the third postnatal week, 85–90% of the cardiomyocytes are binucleated (41, 213). This degree of binucleation varies somewhat between species; in pigs the percentage of binucleated cardiomyocytes can reach up to 32% (81), while in humans estimates have ranged from 25 to 57% (170, 201). Not unexpectedly, this cell cycle exit is accompanied by a coordinated downregulation of positive cell cycle regulators and upregulation of Rb and cyclin-dependent kinase inhibitors (CdkI) p21 and p27, similar to that seen in skeletal muscle (114, 180, 236, 236, 237). The physiological significance of having cells that are binucleated is unclear, but it has been postulated to be an adaptive response in metabolically active cells where the capacity to generate twice the RNA for protein synthesis might be advantageous.

The amount of DNA synthesis in the adult heart is a controversial subject; labeling experiments using tritiated thymidine or bromodeoxyuridine (BrdU) in rodents indicate that the number of cardiomyocytes entering cell cycle in the normal adult heart is very low (197, 212). Tritiated thymidine incorporation assays revealed that only 0.005% of the ventricular cardiomyocytes show evidence of DNA synthesis in uninjured adult mice hearts (211). Although DNA synthesis does not seem to increase significantly in the injured mouse heart (0.004%) (175), a mitotic index of 0.015–0.08% has been reported in injured human myocardium (13, 106). Thus the intrinsic proliferative capacity of adult cardiomyocytes is quite low.

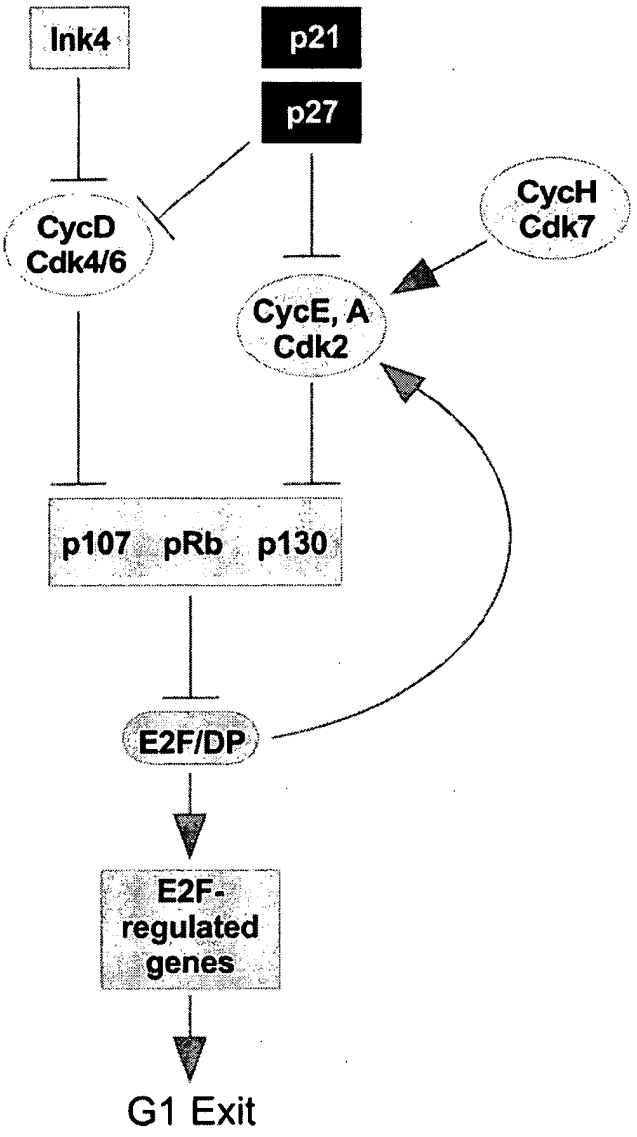

FIG. 1. Regulation of cardiac cell cycle. Schematic diagram of the factors involved in cell cycle progression in cardiac myocytes.

B. Expression and Role of Cell Cycle Regulators During Cardiac Development

Cell cycle progression entails the tightly regulated transduction of mitogenic signals to cyclically expressed proteins known as cyclins and, hence, to their catalytically active targets, the cyclin-dependent protein kinases (Cdks) (Fig. 1). To ensure proper progression through each phase, cells have developed a series of orchestrated checkpoints that govern the different cyclin-Cdk complexes required for distinct cell cycle events. Their activities are regulated by CAK (cyclinH/Cdk7) and Cdk inhibitors (CKI) in both a positive and negative manner, respectively. Key regulators in the major cell cycle checkpoint in late G_1, known as the restriction point in mammalian cells, include Cdk4 and Cdk6, which preferentially assemble into holoenzymes with cyclin D1, D2, or D3 (205). These complexes preferentially phosphorylate members of the Rb family (Rb, p107, and p130) leading to the release of E2F transcription factors. Cyclin E is mainly expressed at the G_1-S transition where it enters into active complexes with its catalytic partner Cdk2 to accelerate the phosphorylation of the Rb proteins. Cyclin A and Cdk2 complexes play a major role in S phase, while cyclin B and Cdc2 are required during G_2/M phase. E2F is required for the transcription of genes involved in mediating G_1 exit and DNA synthesis (163).

1. *Cyclin/Cdk/CdkI*

The embryonic heart exhibits high levels of expression of cyclins involved in G_1, S, G_2, and M-phase like D1, D2, D3, A, B1, and E (22, 73, 109, 250). Additionally, other

genes required for DNA replication such as proliferating cell nuclear antigen (PCNA) and the cyclin-dependent kinases Cdc2, Cdk2, Cdk4, and Cdk6 are also highly expressed along with their associated kinase activities (summarized in Table 1) (22, 73, 109, 250). The relative importance of these factors remains largely unknown, but development of the embryonic ventricle appears to be critically dependent on cyclin D expression. Mice lacking cyclins D1, D2, or D3 are viable and display only narrow and tissue-specific phenotypes reflecting the highly overlapping pattern of expression of the three D-type cyclins (67). Although mice lacking a single cyclin D subtype did not demonstrate a cardiac phenotype, mice deficient for all three D-cyclins (cyclin $D1^{-/-}$, $D2^{-/-}$, $D3^{-/-}$) died at mid/late gestation secondary to heart abnormalities and defective hematopoiesis (117). Mutant embryos displayed severely thinned ventricular walls, mainly affecting the compact zone and large ventricular septal defects. Interestingly, cell cycle kinetics were not abnormal in all tissues, suggesting a tissue-specific utilization of these proteins. Conversely, transgenic mice overexpressing cyclin D1, D2, or D3 in adult myocardium exhibited elevated rates of cardiomyocyte DNA synthesis at baseline in the adult hearts (175). Consistent with this finding, $Cdk2^{-/-}$ and $Cdk4^{-/-}$ mice die during embryogenesis around E15 as a result of heart defects (16). The loss of Cdk2 and Cdk4 caused hypophosphorylation of Rb, which led to repression of E2F target genes, like Cdc2 and cyclin A2. Hearts from these double-mutant mice displayed reduced global size, enlargement of atria, and thin ventricular walls. At the microscopic level, proliferation in some areas of the heart was decreased in mutant compared with wild-type mice. Interestingly, it has been shown that combined loss of Cdk4 and Cdk6 does not affect cell cycle initiation and progression, suggesting that Cdk2 compensates for the lack of cyclin D-dependent kinases (144). These results argue for a specific role for Cyc D/Cdk4 complexes in normal cardiac development.

The withdrawal of postnatal cardiomyocytes from the cell cycle is linked with a change in the expression pattern of many cell cycle regulatory molecules (summarized in Table 1). The protein expression profiles of cyc-

TABLE 1. *Expression patterns of cell cycle regulatory proteins during cardiac development*

Gene	Function	Cell Cycle Phase	Expression Level			Reference Nos.
			Fetal	Neonatal	Adult	
		Cell cycle mediators				
Cyc D1	Cdk4 and −6 partner	G_1/S	++	+	−	108,109,213
Cyc D2	Cdk4 and −6 partner	G_1/S	++	+	−	108,109,214
Cyc D3	Cdk4 and −6 partner	G_1/S	++	+	−	108,109,214
Cyc E	Cdk2 partner	G_1/S	+	+	−	108,250
Cyc A	Cdk2 partner	S and G_2/M	++	+	−	108,250
Cyc B	Cdc2 partner	G_2/M	++	+	−	32,108,250
Cdc2	Promotes G_2/M transit	G_2/M	++	+	−	108,109,136,214
Cdk4	Phosphorylates Rb family members	G_1/S	++	+	−	108,109,213
Cdk2	Phosphorylates Rb family members	G_1/S	++	+	−	108,109,214
Cdk6	Phosphorylates Rb family members	G_1/S	++	+	−	22
		Cell cycle inhibitors				
p16	Inhibits Cdk4 and −6 activity	G_1/S	ND	−	ND	5,114
p18	Inhibits Cdk4 and −6 activity	G_1/S	++	++	−	23
p21	Inhibits Cdk2,−4, and −6 activity	G_1/S	+	+	++	180
p27	Inhibits Cdk2, −4, and −6 activity	G_1/S	+	+	+	114,180
p57	Inhibits Cdk2, −4, and −6 activity	G_1/S	++	−	−	114,159
p53	Regulates cell cycle checkpoint	G_1/S or G_2M	ND	ND	−	93
p193	Regulates G_1/S checkpoint	G_1/S	++	+	−	229
RB	Inhibits E2F activity	G_1/S	−	+	++	101,142
p107	Inhibits E2F activity	G_1/S	++	+	−	101,142
p130	Inhibits E2F activity	G_1/S	+	++	+	101,142
TSC2	Regulates p27 activity	G_1/S	+	++	++	213
		Transcription factors				
E2F1	Transcription factor for cell cycle genes	G_1-S-G_2M	++	+	−	142,235
E2F3	Transcription factor for cell cycle genes	G_1-S-G_2M	++	+	−	235
E2F4	Transcription factor for differentiation	G_0	−	+	++	142
			++	+	−	235
E2F5	Transcription factor for differentiation	G_0	−	+	++	142,235
E2F6	Transcription factor for cell cycle genes	G_1-S	++	+	−	155
N-Myc	Transcription factor for cell cycle genes	G_1-S-G_2M	++	+/−	−	156
c-Myc	Transcription factor for cell cycle genes	G_1-S-G_2M	++	+	−	142,202

++, Relatively stronger expression than +; −, not detected; ND, not determined.

lins D1, D2, D3, A, B1, and E and their associated kinases are significantly downregulated in cardiomyocytes after birth compared with the levels observed in the embryonic heart. Moreover, the protein levels of cyclin A, B, D1, E, and Cdc2 become undetectable by immunoblotting in adult cardiomyocytes (22, 73, 109, 250). The downregulation in expression of cyclins and Cdks during normal development of cardiomyocytes has been shown to parallel a reciprocal upregulation of CdkIs. Two protein families exist to specifically inhibit Cdks. One family of Cdk inhibitors is specific for Cdk4/6 (the INK4 family, comprising p15, p16, p18, and p19); a second, the Cip/Kip family (p21, p27, and p57), has much broader activity inhibiting Cdk4/6 as well as Cdk2 and Cdc2 (205). INK4 proteins selectively inhibit the activity of cyclin D-dependent kinase by competitively binding to these kinases and thus preventing cyclin D interaction. Activity of cyclin E and cyclin A-dependent kinases are negatively regulated by the second CKI family including p21, p27, and p57 (206). Members of this family are also potent inhibitors of cyclin D-dependent kinases and thus have much broader substrate specificity than the INK4 family (184).

Expression patterns of CdkIs in the heart have been characterized by numerous investigators both during normal development and in diseased states. p16 and p18 of the INK4 family have been reported in the embryonic heart, although levels in young adult hearts are low or undetectable (5, 23, 114). It has been suggested that there is a progressive increase in the percentage of p16 positive myocytes with age, which may represent not just quiescent but instead a marker of cardiac myocyte senescence (107, 118). In contrast, expression of Cip/Kip family members, p21 and p27, increases in cardiac myocytes in the perinatal period and reaches high levels in adult myocardium (24, 133). Expression at the transcriptional level of the third member of the Cip/Kip family, p57, has been reported (149), but the protein is detectable in the heart only at early stages in rat, although it persists throughout life in humans (24). Both p21 and p27 are downregulated in cardiac myocytes in response to pathological stressors such as pressure overload, although the significance of this finding remains to be determined (133).

2. Pocket proteins (Rb, p130, p107)

The primary target of G_1 Cdks is the product of the retinoblastoma susceptibility gene (Rb). This protein along with p107 and p130 comprise a family of proteins often called pocket proteins (38). All three family members are expressed in developing myocardium, although the temporal pattern diverges considerably. Rb is scant or undetectable in fetal mouse myocardium at age E12.5, but is upregulated by the neonatal stage, and in adult, terminally differentiated cardiac tissue becomes the predominant pocket protein expressed (101, 142). p107 is expressed in a pattern reciprocal to Rb, highest in the embryonic heart and lowest in adult. p130 expression peaks in the neonatal period and is subsequently downregulated and expressed at low levels in adult myocardium. These proteins are best known for their roles in inhibiting cell cycle progression through the regulation of E2F-responsive genes (42). In their hypophosphorylated form, Rb proteins bind to E2F complexes, recruiting transcriptional repressors such as histone deacetylases (HDACs) or the Jumonji, a repressor that plays a critical role in embryonic heart development (104). Phosphorylation of Rb and its relatives by Cdk2 and -4 results in the release of E2F complexes, enabling them to activate transcription and trigger the expression of genes required for DNA synthesis and further cell cycle regulating molecules such as cyclin E, cyclin A, the mitotic kinase Cdc2 (p34/Cdk1), and E2F-1 itself.

There is accumulating evidence that Rb proteins play a critical role in regulating cell cycle exit and possibly cardiac muscle differentiation. Rb-null embryos die at day 14.5 post coitum with widespread cell death and aberrant cell cycling in a variety of tissues (37, 99, 125) but apparently normal hearts. It was reported recently the $Rb^{-/-}$ embryonic stem cells displayed a delay in the expression of cardiac-specific transcription factors and subsequently cardiac differentiation. It was shown by other investigators that LEK1, a murine homolog of the cardiomyogenic factor 1, interacts with Rb, inhibiting its activity and allowing cardiac myocytes to proliferate despite the presence of Rb proteins during development (9). ES cells deficient in LEK1 recapitulated the delay in cardiac differentiation seen in $Rb^{-/-}$ ES cells (172).

Unlike Rb-deficient mice, p107 and p130 nullizygous mice were initially reported as viable and phenotypically normal (43, 126), but an additional level of complexity was added to the interpretation of pocket protein function by the report of a new p130-deficient mouse strain that although well tolerated in a C57BL/6J background, resulted in embryonic lethality in a Balbc/J background. These mice developed a thin-walled, hypoplastic myocardium with defective looping and chamber formation (124). While these experiments seem to suggest that p130 has a unique, strain-dependent role in cardiac development, p130-deficient mice created by separate investigators (43) do not display a similar phenotype in a Balbc/J background (S. Mao and W. R. MacLellan, unpublished data). The explanation for this discrepancy remains to be determined, although the presence of a modifier gene, unique to original ES cells, might explain this finding. We have demonstrated that Rb and p130 have overlapping functional roles in vivo to suppress cell cycle activators and maintain quiescence in postnatal cardiac muscle. Mice that are deficient in both Rb and p130 demonstrated a threefold increase in the heart weight-to-body weight ratio and showed increased numbers of BrdU- and phos-

phorylated histone H3-positive nuclei, consistent with persistent myocyte cycling (142). These data support the notion that Rb family members are critical for normal cardiac myocyte cell cycle exit but do not address the issue of whether they also mediate the inability of adult myocytes to divide in response to growth stimuli. Studies utilizing temporally regulated, cardiac-restricted Rb null are in progress to resolve this question.

C. Growth Factors and Cytokines

An exhaustive review of the numerous growth factors that have been implicated in mediating cardiac myocyte proliferation is beyond the scope of this review. Instead, we will highlight those signaling pathways for which the most data exist to support a direct role in regulating cardiac growth.

1. Insulin-like growth factor I

Insulin-like growth factor I (IGF-I) is a single-chain polypeptide that has a structural homology with proinsulin. IGF-I has insulin-like short-term metabolic effects and growth factor-like long-term effects on cell proliferation and differentiation of various cell types. IGF-I's mitogenic activity is mediated primarily through binding the IGF-I receptor, also known as the type 1 IGF receptor (130). Mice carrying null mutations of the genes encoding IGF-I and/or IGF-I receptor demonstrate embryonic and postnatal growth retardation without a specific cardiac phenotype and die perinatally of respiratory failure (10, 138). Mice expressing reduced levels of IGF-I (~30% of normal) (129) survive to adulthood and have an intermediate size compared with wild-type or IGF-I-deficient animals. Despite this finding, they display normal left ventricular (LV) mass when normalized for body weight, and normal adaptive hypertrophic response to increased hemodynamic load produced by supra-aortic banding (129). Conversely, cardiac overexpression of IGF-I in transgenic mice shows an increase in heart weight by 50% mediated by an increased number of cells in the heart (187). A similar phenotype was seen in mice lacking the IGF-II receptor, which normally functions to bind and inactivate IGF-I (120). This observation is consistent with in vitro evidence suggesting that IGF-I modulates myocyte proliferation rather than hypertrophy (105). However, a study has also been reported where overexpression of a local form of IGF-I in the hearts of transgenic mice specifically induced hypertrophic phenotype that eventually led to a reduced systolic performance (54). It is not clear if these discrepancies are related to differences in the temporal pattern of gene expression in the transgenics, levels of expression, or simply technical differences. Certainly the signaling components downstream of the IGF-I receptor have been implicated in cardiac hypertrophy by a number of investigators, and the IGF-phosphatidylinositol 3-kinase (PI3K)-Akt-p70S6K signaling pathway has been shown to play an important role in regulating cardiac hypertrophy, viability, and homeostasis (154). Overexpression of an activated form of PI3K catalytic subunit resulted in cardiac hypertrophy, while forced expression of a dominant negative PI3K produced smaller hearts and individual fibers (207). The role of Akt, the downstream effector, is less clear. Several investigators have demonstrated that overexpression of Akt in the heart results in increased heart size secondary to cardiac myocyte hypertrophy (44, 45, 208). In contrast, targeting Akt to the nucleus led to an increased number of cardiomyocytes, smaller in volume with enhanced ventricular function and myocyte contractility (194). However, since overexpressing a dominant-negative Akt during development did not affect cardiac myocyte size or number, Akt may not play a role in normal cardiac myocyte proliferation (207). Recently, it was reported that in IGF-I transgenic mice, cardiac stem cell division is increased, which is accompanied by enhanced telomerase activity, delayed senescence, and preservation of a reservoir of functionally competent cardiac stem cells (227). Given that exogenous delivery of IGF-I has been shown to have beneficial effects postmyocardial infarction (232), IGF-I may provide a useful tool to enhance myocardial repair after injury.

2. gp130-dependent signals

Accumulating evidence supports the concept that cytokines, particularly those that signal through receptor complexes containing the gp130 subunit, play a key role in regulating cardiac myocyte proliferation. These cytokines, which include interleukin (IL)-6, IL-11, leukemia inhibitory factor (LIF), cardiotropin-1, ciliary neurotrophic factor (CNTF), and oncostatin M, have been implicated in both normal and pathological cardiac growth (87, 223). Mice deficient for these factors or their receptors [IL-6 (115), LIF (64, 218), CNTF (148), IL-11Rα (189)] developed a plethora of developmental defects, although none demonstrated specific cardiac defects. In contrast, germline deletion of gp130 results in embryonic lethality with a hypoplastic ventricle, suggesting gp130-dependent signals are critical for proliferative cardiac growth (249). Deleting gp130 in the perinatal period likewise results in myocardial thinning, presumably from a defect in cardiac myocyte proliferation or survival (18). Cardiac specific gp130-deficient mice develop normally with unremarkable appearing hearts at baseline, although hemodynamic stress leads to the development of a dilated cardiomyopathy secondary to increased apoptosis (88). Thus gp130-mediated signaling in cardiac myocytes is not necessary for normal cardiac development, but instead is necessary

for myocyte survival during hypertrophic growth in the adult ventricle. These data raise the interesting possibility that the effects of gp130 deletion in utero are mediated indirectly, possibly through paracrine mechanisms by nonmyocytes in the developing ventricle.

3. Fibroblast growth factor

Fibroblast growth factor (FGF)-2 is a multifunctional protein that plays a vital role in regulating growth and differentiation of various cell types including cardiomyocytes. FGF-2 exerts many of its effects by binding to high-affinity cell surface receptors (FGFR) of the tyrosine kinase family (65), among which the FGFR-1 isoform is the predominant in cardiomyocytes of all developmental stages (110). FGF-2 has been demonstrated to stimulate the proliferation of embryonic and neonatal cardiomyocytes in vitro (173), as well as cardiac stem cells (12). However, other groups have reported that FGF-2 does not control the number of stem cells but regulates their differentiation into cardiac myocytes (191). Blocking FGF signaling in the embryonic heart inhibits cardiac myocyte proliferation (152). Early attempts to directly assess the role of this receptor in cardiac development were hampered by the fact that germline FGFR1-deficient embryos display severe early growth defects (55, 246). Ornitz et al. (122) demonstrated recently that epicardial and endocardial FGF signaling is essential for myocardial proliferation and differentiation in vivo, acting redundantly through FGFR-1 and FGFR-2. Embryos deficient for both FGFR-1 and FGFR-2 specifically in the myocardium displayed severe hypoplasia, enlarged atria, and thinning of the ventricular wall (122). This confirms the functional requirement for both FGFR1 and FGFR2 in developing myocardium and the critical role of FGF signaling for myocardial proliferation.

D. Transcription Factors

1. E2Fs

E2F actually represents a family of transcription factors with eight members, E2F-1 through -8 (140). To bind DNA and activate transcription, E2Fs must form obligate heterodimers with members of a second family of transcription factors, DP-1 and -2. While relatively little is known regarding the specific roles of individual family members in the heart, based on structural and functional characteristics they are often subdivided into activator E2Fs (E2F-1, E2F-2, and E2F-3) and repressor E2Fs (E2F-4, E2F-5, E2F-6, E2F-7, and E2F-8). This classification is based on differences in the ability of these overexpressed proteins to activate transcription or drive quiescent cells into the cell cycle, as well as on the phases of the cell cycle where the E2F proteins can be shown to be present at E2F-regulated promoters. The distinction between these two groups is not absolute. Repressor E2Fs can activate transcription when overexpressed, and activator E2Fs can potentially form complexes with repressor proteins.

E2F-1, -2, and -3 share structural similarity and are expressed in proliferating cells (127) and preferentially associate with Rb (84). In contrast, E2F-4 shares homology with E2F-5, and both are expressed primarily in quiescent and differentiating tissues (48). E2F-4 has been found in complexes with Rb, p107, and p130 (153). E2F-5 binds preferentially with p130 (86). E2F-6, -7, and -8, the newest members, also act as transcriptional repressors, but their physiological role remains undetermined. Ectopic expression of E2F-1, -2, and -3 drives quiescent cells through G_1 into S phase (53, 102, 183), but only E2F-1 induces apoptosis (220). In contrast, the repressive E2Fs, E2F-4 or E2F-5, promote differentiation (177) and are associated with the pocket protein-dependent downregulation of a number of genes involved in cell cycling including E2F-1 (75), cdc2 (226), and b-myb (15). These results suggest that E2F family members and pocket proteins form a complex positive and negative regulatory network regulating proliferation and differentiation.

Limited in vivo data exist for a specific role of any particular E2F family member in cardiac development. E2F-1, E2F-3, and DP family members are downregulated from fetal to adult stages of ventricular development (142). Our data suggest that E2F-4 and -5 are upregulated in heart during development (142); however, others have reported that only E2F-4 is expressed in differentiated cardiac myocytes (235). Hence, the exact expression pattern of E2F-4 in the heart remains unresolved, although upregulation of E2F-4 and -5 has also been seen in skeletal muscle differentiation (46). Deleting E2F-1, -4, or -5 did not result in any discernable cardiac defect (188, 247), perhaps related to functional redundancy existing in this family (72, 94, 137). In contrast, deleting E2F-3 caused embryonic lethality in the majority of embryos, but some mice survived to adulthood, eventually dying prematurely with signs of congestive heart failure (40). Unfortunately, the mechanism for the cardiac failure was never determined. Since the defects in E2F-3 null mice are distinct from those arising in E2F-1 mutant mice, it suggests that activating E2Fs must have unique biological functions in vivo. Since it required the combined loss of all three activating E2F family members (E2F-1, -2 and -3) to abolish the ability of cells to enter S phase and proliferate (242), it is not surprising that cardiac myocyte proliferation did not appear to be affected in any of the E2F knockouts. Overexpression of E2F-1, -2, -3, and -4 in neonatal cardiomyocytes can induce S-phase entry, but increased expression of E2F-1 and E2F-3 induced apoptosis along with cell cycle progression (59). These results are similar to previous reports demonstrating that E2F-1-

induced DNA synthesis and apoptosis in adult myocardium (2) and that E2F-1 is the family member most associated with apoptosis (72, 199). Thus the specific role of E2F members in cardiac development and proliferation remains unresolved.

2. Myc

Myc is the prototypical member of a family of sequence-specific DNA-binding proteins that are postulated to act as "third messengers" for ligand-dependent signals and are implicated in the regulation of growth in a variety of tissues (66). The Myc family, which includes c-Myc, N-Myc, and L-Myc, is a group of transcription factors of the basic helix-loop-helix-leucine zipper (bHLHZ) family of proteins that activate transcription as part of a heteromeric complex with a protein termed Max. Family members share a common binding motif and have been demonstrated to be capable of functionally complementing each other, suggesting the Myc gene family must have evolved to facilitate differential patterns of expression (145). Although there is a consensus that Myc is critical for normal development, the mechanism whereby it exerts this effect is controversial. Studies in vivo, using Myc transgenics and conditional knockouts of Myc, have concluded that Myc is critical for both proliferation and cellular growth (52, 96), while others have implicated Myc in cellular division alone (228). In the heart, evidence exists to support a role for Myc in both cardiac myocyte division and hypertrophy as summarized below.

Myc is expressed in embryonic ventricular myocytes, and Myc-deficient mice die prematurely at E10.5 with cardiac defects (51). Myc-null embryos were generally smaller and retarded in development compared with their littermates, suggesting a general role for Myc in cellular proliferation, and displayed heart enlargement and pericardial effusions. Unfortunately, a detailed analysis of the cardiac phenotype was never performed. Therefore, no data exist at a cellular level as to the effects of the Myc deficiency on cardiac myocyte size or number. Likewise, whether the observed myocardial defects are due to a primary effect of Myc deficiency on myocytes versus an indirect effect secondary to Myc's effects on other organ systems is unknown. Conversely, transgenic mice that overexpressed Myc in the fetal myocardium developed ventricular enlargement secondary to myocyte hyperplasia (100). Despite the hyperplasia, postnatal DNA synthesis ceased earlier in transgenics than in wild-type mice (141). Although initially counterintuitive, it is now known that overexpression of protooncogenes such as Myc in primary cells can result in cell cycle arrest through the induction of $p19^{ARF}$ (69). These data support the notion that Myc is sufficient to mediate hyperplastic cardiac growth.

3. Hypoxia inducible factor-1α

Hypoxia inducible factor-1 (HIF-1) is a transcription factor complex consisting of α- and β-subunits that heterodimerize to form a functional complex, which activates or represses genes containing hypoxic response elements. HIF-1 is stabilized and activated in response to hypoxia and/or activation of specific signaling cascades. In contrast to HIF-1β, which is constitutively expressed and functions also as a dimerizing partner for transcription factors not involved in hypoxia, HIF-1α is unique in its sensitivity and specificity to changes in oxygen levels. HIF-1α stability is regulated by the van Hippel-Lindau (VHL) tumor suppressor protein. In the absence of VHL, HIF-1α is not targeted for proteasomal degradation and is thus stabilized (47, 215). Deletion of HIF-1α resulted in developmental arrest and lethality by E11 in HIF-$1\alpha^{-/-}$ embryos with multiple defects in cardiovascular development, including pericardial effusion and disorganized cardiac morphogenesis with myocardial hyperplasia and ventricular obstruction. The hyperplasia of cardiac myocytes resulted in a constriction between the ventricle and outflow tract of HIF-$1\alpha^{-/-}$embryos (97). There was also a reduction in vascularization that was attributed to decreased expression of vascular epidermal growth factor (VEGF), an angiogenic factor that is known to be induced by tissue hypoxia via HIF-1α. However, since the increase in cardiac cell number seen in HIF-$1\alpha^{-/-}$embryos was dramatically different from the hypoplastic myocardium described previously for $VEGF^{+/-}$ mice, the myocyte hyperplasia was unlikely secondary to impaired VEGF production or vasculogenesis. Instead, it argues for a more direct role for HIF-1α in cardiac proliferation and morphogenesis.

Although numerous genetic studies have indicated the requirement of HIF-1α for hypoxia-induced growth arrest and activation of p21, a role in regulating development cell cycle arrest is a novel function of HIF-1α. The mechanism underlying HIF-1α-induced growth arrest has been elusive, but it has been shown that even in the absence of a hypoxic signal, HIF-1α can induce cell cycle arrest by functionally counteracting Myc, thereby derepressing p21 (116). The HIF-1α-antagonism is mediated by displacing Myc binding from the p21 promoter. Interestingly, neither HIF-1α-transcriptional activity nor its DNA binding was essential for cell cycle arrest, indicating a divergent role for HIF-1α. Additional studies have also supported the hypothesis that stabilization of HIF-1α inhibits proliferation. For example, HIF-$1\alpha^{-/-}$ tumors grow faster and become more invasive than its wild-type counterpart (44, 230). Furthermore, HIF-1α stabilization not only fails to promote, but actually decreases tumor growth (108). HIF-1α, similar to Myc, has also been implicated in playing a role in cardiac hypertrophy (36,

112). A comprehensive analysis of the role of HIF-1α in cardiac proliferation and hypertrophy still needs to be performed, but given Myc's critical role in mediating cardiac myocyte growth, HIF-1α may represent a novel endogenous antagonist.

E. Signaling Pathways

1. Tuberous sclerosis complex 1 and 2

Tuberous sclerosis complex (TSC) is an autosomal dominant tumor syndrome characterized by the appearance of tumorlike growths that affect many organs including the heart. The syndrome often causes seizures, mental retardation, and a variety of developmental disorders, including autism (119). The TSC disease-causing genes have been identified and encode for proteins called hamartin (234) and tuberin (225a), respectively. Myocardial tumors are rare phenomena in humans, but >50% of TSC patients show evidence of primary myocardial tumors (239). This propensity for cardiac tumor formation in TSC patients suggested that the TSC gene products could play an important role in the regulation of cardiomyocyte cell cycle. Eker rats, heterozygous for a germline mutation in TSC2 ($TSC2^{EK/+}$), are predisposed to renal carcinoma, and animals homozygous for mutation ($TSC2^{EK/EK}$) die in utero (171). Embryonic cardiomyocytes isolated from $TSC2^{EK/EK}$ embryos on E12.5 continued to actively proliferate and synthesize DNA after as many as eight passages in contrast to those from heterozygous or wild-type embryos, which exited the cell cycle. Because $TSC2^{EK/EK}$ cardiomyocytes retained a highly differentiated phenotype similar to normal embryonic or neonatal rat cardiomyocytes, it was concluded that the TSC2 gene product is important specifically for normal cardiomyocyte cell cycle withdrawal and terminal differentiation. Overexpression of a mutant TSC2 in the hearts of transgenic mice that is predicted to block the growth inhibitory activity of the endogenous TSC2 resulted in normal cardiac development and cardiac myocyte cell cycle exit. Nonetheless, the level of cardiomyocyte DNA synthesis in transgenic mice was increased 35-fold above that of nontransgenic littermates in response to hypertrophic stimuli (174). TSC proteins are known to be positive regulators of the cyclin-dependent kinase inhibitor p27, by inhibiting its degradation by the ubiquitin-proteasome pathway (193). Accordingly, it was assumed that aberrant trafficking of p27 might be the underlying cause of the altered cell cycle regulation observed in these transgenic mice, but cytoplasmic sequestration of p27 was not seen in transgenic hearts (174). At present, the mechanism underlying the enhanced DNA synthesis in mice expressing the modified TSC2 transgene is not clear. Furthermore, it is not known whether those cardiomyocytes synthesizing DNA did eventually undergo karyokinesis and/or cytokinesis. Identification of pathways activated in the responsive cells might uncover mechanisms to increase cell cycle reactivation to a point sufficient for regenerative growth of the heart.

2. p38 mitogen-activated protein kinase

In mammalian heart, mitogen-activated protein kinase (MAPK) signaling pathways have been hypothesized to regulate cardiomyocyte growth in response to diverse developmental signals (135). The MAPK signaling pathways consist of at least three prominent phosphorylation cascades terminating in the activation of extracellular signal regulated kinases (ERK), c-Jun NH_2-terminal kinases (JNK), or p38 MAPKs. In cardiac myocytes, the ERK cascade is thought to be primarily activated in response to tyrosine kinase receptor and G protein-coupled receptor (GPCR) activation, while the JNK and p38 cascades are activated by both GPCR activation and stress signals. Of the four different p38 isoforms that have been identified, the predominant isoform expressed in the adult heart is p38α, while p38β and p38γ are expressed at low levels, and p38δ is not expressed in the heart (135, 238). The major upstream activators of p38 MAPKs are MAPKKs including MKK3, MMK4, and MKK6, which directly phosphorylate the dual site in p38 MAPKs (Thr-Gly-Tyr). Substrates of p38 MAPKs include mainly other protein kinases and a growing list of transcription factors that includes MEF2, MAPKAPK2 and -3, ATF-2, ELK-1, Chop, TEF-1, C/EBPβ, and Max (6, 251). While it is known that p38 induces differentiation, its role in proliferation has also been recently recognized in many cell types (7, 161, 243). Depending on the cell type and stimulus, p38 MAPKs can have either a positive or negative influence on cell cycle progression (161). p38 appears to be required for proliferation of Swiss 3T3 cells induced by FGF-2 (143), for proliferation of hematopoietic cells induced by granulocyte colony stimulating factor (185), and for proliferation of erythropoietin-dependent cell line FD-EPO (160). On the other hand, in CCL39 and NIH 3T3 fibroblasts, p38 inhibits cell cycle progression at the G_1/S transition, possibly by the inhibiting cyclin D1 expression (123). Recently, it was suggested that p38 could also serve as a key negative regulator of the mammalian cardiomyocyte cell cycle. Genetic activation of p38 in vivo reduced fetal cardiomyocyte proliferation, whereas targeted disruption of p38α along with growth factor stimulation of cultured adult myocytes promoted cardiomyocyte cell cycle reentry. This study demonstrated a modest increase in mitotic cardiac nuclei index of 0.14% (62). However, in this study the adult myocytes were cultured in vitro for 12 days, which results in cardiac myocyte dedifferentiation and recovery of proliferative potential (39). More recently, the

same authors demonstrated that treatment with FGF1 in combination with a p38 MAPK inhibitor after myocardial injury led to an increase in mitotic index in vivo, suggesting a role for this pathway in maintaining terminal differentiation in addition to regulating cardiac cell cycle (61). Complicating the interpretation of p38α's role in adult myocardium is a report that cardiac-specific transgenic mice expressing a dominant negative mutant p38α generates a hypertrophic rather than proliferative response in adult hearts (20). Thus further characterization of p38α mutant mice and its molecular interaction with growth factor signaling will be necessary to clarify p38's role in cardiomyocyte proliferation and terminal differentiation.

III. REGULATION OF CARDIAC MYOCYTE CELL CYCLE EXIT

A. Adult Cardiac Myocytes Are Terminally Differentiated

Many differentiated tissues undergo cell cycle arrest as part of their differentiation pathway, but not all cells undergo permanent arrest, and notable examples exist of highly specialized cell types having the capacity for regeneration (68). Terminal differentiation invariably involves two closely linked phenomena: permanent withdrawal from the cell cycle and cell type-specific differentiation characterized by the upregulation of a panel of tissue-specific genes. For instance, fetal or neonatal rodent cardiac myocytes primarily express β-myosin heavy chain (MHC) and skeletal actin, but as cardiac myocytes undergo the process of terminal differentiation, they are downregulated and α-MHC and cardiac actin are upregulated in their stead (222). Consequently, although often used interchangeably in differentiated cell types, cell cycle exit and terminal differentiation are not synonymous. We define a terminally differentiated cell type as one where the majority of cells do not reenter the cell cycle in response to mitogens or normal physiological stress.

Interestingly, although cell cycle reentry occurs rarely in the adult mouse myocytes in response to stress or injury to any significant degree (210), there is accumulating evidence that it likely does to a limited extent in the adult human heart (13). Consistent with this finding, data have existed for some time documenting increased DNA content per nuclei and nuclei per myocyte in cardiomyopathic human hearts (14, 80, 85). However, while restricted cell cycle reentry may occur in the injured human versus mouse hearts [1–4% (13) human vs. 0.0014% mouse (210) cardiac myocytes], the ultimate fate of these myocytes and whether species-specific differences really exist with respect to proliferative capacity remain unresolved. In contrast, in species such as newt where consensus exists on the capacity for myocardial regeneration, it has been demonstrated that mononucleated myocytes were more likely to successfully undergo cytokinesis than binucleated myocytes (150). When binucleated newt myocytes enter the cell cycle, it resulted in variably nucleated myocytes in the majority of cases as opposed to cytokinesis. Thus the reported difference in the percentage of mono- versus binucleated cardiac myocytes between species (41, 81, 170, 201, 213) may account for some of the differences in potential for cell cycle reentry that have been reported. This may be due to the presence of a "tetraploid checkpoint" that arrests cells that fail to undergo cytokinesis in the following G_1, which may be invoked in binucleated myocytes (221). Even in humans, the species with the highest percentage of mononucleated cardiac myocytes and reported cell cycle reentry, significant regeneration does not seem to occur after injury. Others have suggested the critical factor that determines adult cardiac myocyte proliferation is not the nucleation state, but rather its cellular size. It has been reported that myocytes smaller in size retain higher potential to reenter into the cell cycle than the fully differentiated myocytes in response to stress or injury in the adult heart (13, 106, 233).

Although Beltrami et al. (13) concluded that their finding of mitotic nuclei in a minority of cardiac myocytes in the adult failing heart represented cardiac myocyte division, an equally plausible explanation is that the DNA synthesis or nuclear mitosis they observed simply resulted in endoreduplication (increased DNA per nuclei or increased nuclei per myocyte) as others have reported in human myocardium (85) without actual cardiac myocyte division. Consistent with this, studies subsequently have suggested that entrance of human cardiomyocytes into the cell cycle after myocardial infarction is transient and limited and that as opposed to cytokinesis and proliferation, it leads to endoreduplication (151). An increase in ploidy and nuclei per myocyte can also be seen in adult mouse myocardium after genetic manipulation (245). Endoreduplication may account for the discordance between the observed regenerative capacity of the heart after injury and that proposed based on pathological examination of cycling myocytes. Presently, no convincing evidence exists for the formation of a contractile ring in adult cardiac myocytes of any species, which would be a necessary requirement for any cytokinesis.

A third explanation for the observation of cycling myocytes in adult myocardium has also recently been proposed (192, 231). Investigators have identified and characterized several cardiac progenitor cells capable of proliferating (12, 168). However, once committed to the myocyte lineage, the progeny of a stem cell can only undergo three to four rounds of cell division before permanently withdrawing from the cell cycle (12). Thus adult

hearts, regardless of species, are likely composed of predominantly terminally differentiated myocytes that do not reenter the cell cycle, with a minority of myocytes or resident stem cells that are capable of some limited cell cycle reentry. Regardless, neither adult cardiac myocytes nor cardiac stem cells seem to possess the proliferative potential to regenerate the heart after injury such as myocardial infarction.

B. Basis for Cell Cycle Exit and Terminal Differentiation in Cardiac Myocytes

Despite others' and our work, the mechanisms underlying the cell cycle exit and the permanent growth arrest in cardiac muscle are poorly understood. Rb has been implicated in mediating not only cell cycle exit, but also the irreversibility of cell cycle arrest associated with terminal differentiation in various lineages including skeletal muscle (82), adipocytes (34), and macrophages (33). Early studies in vitro using fibroblasts induced to transdifferentiate into skeletal myocytes by overexpression of MyoD demonstrated that $Rb^{-/-}$ but not p107- or p130-null skeletal myocytes have a defect in cell cycle exit and maintenance of quiescence (164, 165). Thus Rb appeared uniquely required for normal myogenic cell cycle control and full differentiation. These results were confirmed in vivo by deleting a floxed Rb allele either in proliferating myoblasts or after differentiation (92). Deleting Rb before myogenic differentiation with Myf5-Cre resulted in a severe defect in differentiation and apoptosis. If Rb was deleted after differentiation, the cells formed normal multinucleated myotubes that did not enter S phase in response to serum stimulation. It was subsequently shown that serum did not induce DNA synthesis in differentiated myotubes even if all three pocket proteins had been removed (28). The authors concluded that Rb plays a crucial role in the switch from proliferation to differentiation in skeletal myocytes rather than maintenance of the terminally differentiated state. Studies using mouse inner ear hair cells, a terminally differentiated cell type, showed that Rb is required for maintaining quiescence in differentiated cells (146). Acute Rb loss resulted in fully differentiated hair cells of the inner ear reentering the cell cycle and proliferating. Thus Rb's role in maintaining terminal differentiation may be tissue specific.

Cardiac-restricted Rb-deficient mice, where Rb was deleted in differentiated cardiac myocytes using an α-MHC-driven Cre transgene, develop normally and do not display cardiac cell cycle defects even after physiological and pharmacological growth signals (142). Because $Rb^{-/-}$ embryonic stem cells display a delay in cardiac differentiation, a developmental specific dependence on Rb may be operable in cardiac myocytes similar to skeletal muscle (172). Rb may be necessary for commitment and differentiation of cardiac myocytes, but once differentiated it is dispensable. When the other pocket protein expressed in adult myocardium, p130, was also deleted, the resultant Rb-p130-null mice displayed defects in cardiac myocyte cell cycle exit and differentiation (142), demonstrating that in cardiac muscle, Rb and p130 clearly have overlapping roles in mediating cardiac myocyte cell cycle exit. Whether Rb and p130 are also necessary for maintaining quiescence in cardiac myocytes has yet to be determined. These results likely explain the observation that cardiac-specific transgenic mice with increased Cdk4 (175, 214) or Cdk2 (136) activity resulted in an increase in cardiac myocyte number and ongoing DNA synthesis in adult hearts, since these maneuvers would be predicted to inactivate all pocket proteins not just Rb.

C. Regulation of Cytokinesis

Cytokinesis is the final step of cell division. It is responsible for partitioning and separation of cytoplasm between daughter cells to complete mitosis (95). One strategy to study cytokinesis has been to focus on the regulation of the mechanical components responsible for contraction of the cleavage furrow, namely, the actomyosin cytoskeleton. Actin has been postulated to act as a scaffold onto which the rest of the cytokinesis machinery assembles. Apart from actin and myosin, other possible key players such as small GTPases like RhoA and its effectors ROCK I and ROCK II, citron kinase, formin-homology proteins, GTPase Cdc42, Rac, and septins have been identified in regulating the formation of the contractile ring (77). The formation of the contractile ring has been investigated in postnatal rat cardiomyocytes (131, 132). However, these studies were restricted only to the expression and subcellular localization of F-actin and nonmuscle myosin. In dividing neonatal myocytes, actin gets disassembled during early stages of mitosis and concentrates at the equator of the spindle during anaphase before finally forming an intensely staining, circumferential band in telophase. In contrast, cytoplasmic myosin evenly distributes in the cytoplasm as small spots, concentrates in association with the cortical membrane in the equator region in anaphase, forms a ringlike structure in telophase, and remains associated with adjacent membranes at the cleavage furrow until telophase (131). The study could demonstrate the assembly of actin and myosin at the contractile ring during the binucleation process, and authors suggested that the molecules involved in the later part of cytokinesis may be responsible for the

binucleation of cardiac myocytes during postnatal development. None of the other proteins involved in cytokinesis was analyzed with the exception of Polo-like kinase, a protein involved in spindle formation and chromosome segregation during mitosis. It was shown to be downregulated in the adult heart (76). More recently, the localization of anillin, a known regulator of the cleavage furrow formation, was characterized in dividing versus binucleating cultured cardiomyocytes. It was reported that the failure to undergo abscission, which leads to binucleation, is due to defective focusing of anillin in the midbody region (63). Among the other regulators of cytokinesis, septins, a family of cytoskeletal GTPases important for cytokinesis, have been shown to demonstrate stage-specific expression during heart development (4). Likewise, cardiomyocytes display a developmentally regulated expression of small Rho GTPases such as RhoA, Cdc42, Rac1, ROCK-I, ROCK-II, and *p*-cofilin that are coupled to the formation of actomyosin ring. High levels of these proteins were present in embryonic hearts where cytokinesis occurs but were downregulated perinatally as cardiac myocytes exit the cell cycle (P. Ahuja and E. Ehler, unpublished data). The complex myofibrillar cytoarchitecture that develops postnatally, together with the fact that there is downregulation in the expression levels of proteins that regulate cell cycle and cytokinesis, respectively, might contribute to uncoupling of karyokinetic and cytokinetic events as seen in the postnatal cardiomyocytes.

One long-standing theory to explain the lack of cytokinesis in adult cardiac myocytes is the presence of highly organized mature myofibrils in the adult cardiomyocytes which physically prevent cell division. Because cells must disassemble their cytoskeletal filaments before entering cell division, disassembly of the cytoarchitecture in adult cardiac myocytes would presumably negatively impact myocyte contractile function, although this seems to occur in fetal cardiomyocytes, which completely disassemble the myofibrils before dividing (3). This disassembly occurs in two steps with Z-disk and thin-filament-associated proteins getting disassembled before disassembly of the thick (myosin) filaments. Thus the cellular shape and cell-cell contacts of dividing myocytes remain similar to nondividing cells, which may be necessary for uninterrupted function of the working myocardium. In adult cardiomyocytes, the presence of stable, highly ordered, and functional myofibrils may physically prevent cell division. It is known that during progressive differentiation from the embryonic to adult stage, there is a gradual increase in the size, number, and complexity of organization of myofibrils in ventricular cardiomyocytes (60, 89, 196). In contrast, atrial cardiomyocytes are smaller in size, ~40% poorer in myofibrils, and appear less differentiated. Interestingly, they also retain a higher ability to regenerate both in vivo and in vitro (198, 217), which might explain why atrial tumors were more common in transgenic mice expressing a fusion of atrial natriuretic factor and simian virus 40 (SV40) T-antigen (111). Thus the impediment to complete cytokinesis in adult cardiac myocytes remains speculative, but sarcomeric structure likely plays an important role.

IV. CELL CYCLE REGULATORS IN CARDIAC HYPERTROPHY

During development, cell cycle progression is tightly coupled to the accumulation of cell mass (cell growth) to ensure that cell size is constant (162). In contrast, in many human diseases cell growth can become uncoupled from proliferation, resulting in hypertrophic growth. Because cardiac hypertrophy is associated with such negative outcomes, much effort has been focused on characterizing the intracellular signal transduction pathways that are associated with cardiac hypertrophy (58). The link between this process and cell cycle progression as well as whether the same factors that regulate hyperplastic growth also mediate hypertrophic growth in adult postmitotic myocytes have been largely ignored. We review the data linking the aforementioned cell cycle regulators in regulating cardiac myocyte hypertrophy.

A. Cyclin/Cdk/CdkIs

Hypertrophic stimulation of adult myocardium is accompanied by the upregulation of G_1 cyclin/Cdks (134, 179) and reciprocal downregulation of CdkIs (133). Some investigators have interpreted these data as evidence of postmitotic cardiac myocytes reentering the cell cycle and undergoing proliferative growth. Increasing data are accumulating that proteins classically thought to be involved in cell cycle regulation also play a critical role in the control of cellular growth. In *Drosophila*, CycD/Cdk4 regulates cell size and number; however, the effect of CycD/Cdk4 overexpression varied depending on the cell type (50). CycD/Cdk4 stimulates cell growth in postmitotic cells but proliferation in cells capable of cell cycle reentry (50). This CycD/Cdk4-induced cell growth was dependent on a gene encoding the mitochondrial ribosomal protein, mRpL12 (74). In the absence of mRpL12, cells demonstrated reduced growth and mitochondrial activity, suggesting that CycD/Cdk4 controls cell growth via a mitochondrial-dependent pathway. Recently, the same group showed that the orthologous CycD/Cdk4 mammalian complex can stimulate growth in *Drosophila* through a very similar pathway compared with the fly complex (32). A number of reports have implicated CycD/Cdk4 in regulating cardiac hypertrophy in mammalian cells as well, although the downstream effectors have not

been identified (25, 225). Although forced expression of CycD2 led to cell cycle activation and not hypertrophy in cardiac myocytes (26, 175), this might be related to the fact that analogous to the *Drosophila* model (50), the transgene expression in this study began during fetal development when cardiomyocytes are able to proliferate. Inhibiting G_1-cyclin/Cdk activity in the adult, postmitotic cardiac myocytes block hypertrophic growth (166, 224). Consistent with this finding, our laboratory has demonstrated that Myc-induced cardiac hypertrophy is attenuated in CycD2 null mice (252).

A novel mechanism to control cardiac hypertrophy through a novel class of Cdks has recently been proposed (200). This Cdk9 can interact directly with the core transcriptional machinery by phosphorylating the COOH-terminal domain (CTD) of RNA polymerase II, increasing its transcriptional activity. Cdk9 activity was increased in a number of in vitro and in vivo models of cardiac hypertrophy (200). Cdk9 kinase associates with cyclin T1 or T2 and is a component of the transcription positive-acting complex pTEFb, which facilitates the transition from abortive to productive transcription elongation by phosphorylating the CTD of RNA polymerase II. In these hypertrophic models, Cdk9 activation was not related to changes in the level of Cdk9 or cyclin T. Instead, it involved the dissociation of 7SK small nuclear RNA (snRNA), an endogenous inhibitor, from the Cdk 9 complex. In culture, dominant-negative Cdk9 blocked endothelin (ET)-1-induced hypertrophy, whereas an antisense inhibition of 7SK snRNA provoked spontaneous cell growth. In transgenic mice, activation of Cdk9 activity via cardiac-specific overexpression of cyclin T1 is sufficient to provoke hypertrophy. Together, these findings implicate Cdk9 activity as a novel regulator of cardiac hypertrophy.

B. Myc

Myc has been implicated in regulating growth, differentiation, apoptosis, and metabolism in a wide variety of organisms and cell types (176). It is one of the few factors implicated in controlling both cell size and number (219, 228). This is of importance in the heart, since Myc is expressed in embryonic ventricular myocytes and is upregulated in adult myocardium in response to virtually all hypertrophic stimuli (98, 216). Creation of transgenic mice that overexpress Myc in myocardium has led to an evolution in the postulated role of this transcription factor in mediating cardiac hypertrophy. Initially generated transgenic mice overexpressing Myc did not display baseline cardiac myocyte hypertrophy, but hypertrophic growth in adult cardiac myocytes was potentiated in response to some (triiodothyronine), but not all (isoproterenol) agonists (190). This led to investigators questioning the importance of Myc in regulating cardiac growth. To readdress this issue, we created mice where Myc could be inducibly activated, specifically in adult myocardium. Our results demonstrate that Myc activation is sufficient to induce hypertrophic growth in adult myocardium even in the absence of G_1 exit (245). Conversely, we have recently demonstrated that Myc-deficient adult hearts have attenuated stress-induced hypertrophic growth, secondary to a reduction in cell growth of individual myocytes (252). The view that Myc can mediate cellular growth is also supported by the fact that decreased expression of a homolog of c-Myc, dMyc, in *Drosophila* reduces cell proliferation and cell size (103). In contrast, dMyc overexpression leads to an increase in cell size without affecting proliferation rate.

Myc is known to regulate multiple candidate genes implicated with cell growth and metabolism (49, 78). The mechanisms by which Myc regulates cellular growth are less clear; nevertheless, it is interesting to note that the genes responsible for its ability to promote the cell cycle have also been implicated in regulating cell size under certain circumstances. Myc activation in the heart is accompanied by the upregulation of cyclin D2 and cyclin-dependent kinase Cdk2 and Cdk4 activities which are important for cell cycle progression (245). To explore the dependence of Myc-induced cell growth on CycD2, we created bigenic mice where Myc can be selectively activated in CycD2-null adult myocardium. Myc-dependent hypertrophic growth and cell cycle reentry are blocked in CycD2-deficient hearts (252). In contrast to Myc-induced DNA synthesis, hypertrophic growth was independent of Cdk2 activity. These data suggest that Myc is required for a normal hypertrophic response and that its growth-promoting effects are also mediated through a CycD2-dependent pathway. Myc has also been implicated in directly regulating activity of the components of the biosynthetic apparatus. Cardiac hypertrophy is accompanied by a rise in transcription by RNA polymerase III, which produces essential ribosomal components, including 5S rRNA and tRNAs (79). This increase in transcription is achieved by changes in both the activity and level of the essential polymerase III-specific transcription factor TFIIIB. Given that small molecule inhibitors of Myc currently exist (248), targeting of a transcription factor like c-Myc might provide a novel therapeutic approach for inhibiting the development of cardiac hypertrophy and thereby preventing the onset of heart failure.

The mechanisms whereby Myc is activated in cardiac hypertrophy are not clear, but both Myc and cyclin D1 can be activated by β-catenin (83, 209). β-Catenin is a multifunctional protein that can act in the cytoplasm to link cadherins to the actin cytoskeleton or enter the nucleus and function as a transactivator (240). In the absence of

Wnt signaling, free β-catenin is phosphorylated by glycogen synthase kinase-3β (GSK-3β) and rapidly targeted for proteasomal degradation. Numerous hypertrophic signals stimulate a cascade that inhibits β-catenin degradation (58). Stabilization of β-catenin is associated with its translocation to the nucleus, where it interacts with members of the lymphoid enhancer factor (LEF)/T-cell factor (TCF) and activates specific target genes such as Myc and CycD. β-Catenin is upregulated in stress-induced hypertrophy, and targeted deletion of β-catenin in the heart blunts hypertrophy in response to pathological stress (35). Deletion of β-catenin blunts expression of Myc in response to pressure overload, directly linking this signaling pathway to Myc in the regulation of hypertrophic growth in the heart.

C. E2F

Very little is known about the potential roles of these transcription factors in cardiac hypertrophy. E2F-4 and -5 are the predominant E2F family members expressed in adult cardiac myocytes (142, 235). Recently, expression patterns of E2F-6 and E2F-6b in rat cardiomyocytes were characterized, and it was shown that E2F-6 protein is downregulated too developmentally like other E2F proteins and is upregulated during the development of cardiac hypertrophy (155). Although induction of E2F-1, -3, and -4 was observed in neonatal cardiomyocytes treated with serum or phenylephrine, these results should be interpreted with caution given that cultures of neonatal cardiac myocytes are not truly terminally differentiated and it is unknown whether these changes also occur in adult myocardium in vivo subjected to a physiological hypertrophic stress. Nonetheless, it was also shown that inhibition of E2F abrogates the development of cardiac hypertrophy (235). Inhibiting E2F activity with a specific peptide that blocks E2F-DP heterodimerization prevented the induction of hypertrophic markers like atrial natriuretic factor and brain natriuretic peptide, reduced the increase in myocyte size, and inhibited protein synthesis in the cardiomyocytes stimulated with serum and phenylephrine (235). The mechanism whereby E2F might participate in the hypertrophic response is speculative. In skeletal muscle hypertrophy, a subset of E2F-1 target genes involved in protein synthesis, cytoskeletal organization, and mitochondrial function but not G_1 exit have been shown to be upregulated during a hypertrophic response (90). Interestingly, cytochrome-*c* oxidase subunits IV, V, and VIIc are upregulated during the hypertrophic response, and at least cytochrome-*c* oxidase IV is a direct transcriptional target of E2F-1 (90). Given that cardiac hypertrophy is accompanied by an increase in mitochondrial number and activity (244), E2Fs may play an important role in mediating mitochondrial biogenesis and function enabling myocytes to cope with the increased energy demands of the hypertrophic state.

V. POTENTIAL FOR CARDIAC MYOCYTE SELF-RENEWAL

Many mammalian tissues respond to injury by activating committed progenitor cells or stem cells or through proliferation of differentiated cells such as liver or endothelial cells (21). In contrast, adult mammalian cardiomyocytes have very limited potential for self-renewal. On the other hand, it has been known for some time that cardiomyocytes from lower vertebrates are capable of dividing postnatally and regenerating myocardium after injury (17, 150, 158, 167).

A. Cardiac Regeneration in Nonmammalian Models

Amphibians, such as newt, were the first adult vertebrates identified that are capable of regenerating their organs. This ability to regenerate large sections of the body is widespread in Metazoan phylogeny, and this discovery was an important feature of the emergence of experimental biology in the 18th century (57). Although the process of tissue regeneration unfolded in a different manner in the heart, limbs, or tail of the adult newt, they all depend on the plasticity of the remaining differentiated cells after the tissue injury. Zebrafish can also regenerate the heart and other organs like fins, retina, and spinal cord, which has facilitated the application of modern molecular biology techniques to study this phenomenon (182). After the removal of the apical region of the ventricle, the heart in both newt and zebrafish seals by contraction around the clot. Newt and zebrafish adult cardiomyocytes reenter the cell cycle and divide in a zone that surrounds the clot (167, 182). If the animal is injected with labeled thymidine, to identify those cells that are in S phase, ~10% of the cardiomyocytes in this region are labeled in a 1-day period. More recent studies suggest that although cardiomyocytes can enter S phase, more than half of these cells stably arrest at either entry to mitosis or during cytokinesis, similar to what is seen in mammalian cardiomyocytes (17). Only one-third of the cardiac myocytes entering the cell cycle progress through mitosis and enter successive cell divisions. This suggests proliferative potential is retained in only a subset of cardiomyocytes and that regulation of proliferation in the majority is similar to that described for their mammalian counterparts, as they arrest during mitosis or cytokinesis.

The molecular mechanisms that underlie the difference in cardiomyocyte proliferative potential between these species and mammals are unknown. Interestingly, a variant strain of zebrafish with a mutation in checkpoint kinase called Mps1 fails to regenerate its heart after ven-

tricular resection and develops scars in the damaged myocardium (182). These studies suggest that injury stimulates a proliferative response in these species that can lead to myocardial regeneration. No similar response to injury has been observed in the mammalian hearts. It is likely that with the evolution of several organs, including the heart, lost was the ability to regenerate with a corresponding increase in the intricacy of patterning and function. Defining the molecular basis of regeneration in these unique nonmammalian model systems may illuminate basic insights into cardiomyocyte regeneration. Similar to newt and zebrafish, a unique regenerative response was also documented in the MRL mouse strain after a cryoinjury to the myocardium (128). The MRL mouse strain has a dramatically enhanced capacity to heal surgical wounds, a complex trait that maps to at least seven genetic loci. When these mice were subjected to cardiac injury, scarring was markedly reduced and cardiomyocyte mitotic index increased 10-fold in MRL mice compared with C57BL/6 mice. Several other groups have since reported that heart regeneration in the MRL mouse either does not occur or is much more limited than reported previously by Heber-Katz and colleagues in response to experimental myocardial injury (1, 169).

B. Cardiac Myocyte Stem and Progenitor Cells

The dogma that the heart is a postmitotic nonregenerating organ has recently been challenged. Multiple groups have independently described a resident cardiac stem cell or progenitor cell population with the capacity to differentiate into cardiac myocytes (12, 121, 168). The first endogenous cardiac stem cell reported was a Lin^-; c-*kit*$^+$ that was reported to differentiate into cells that are phenotypically indistinguishable from cardiomyocytes in vivo (12). In addition, these clones may also differentiate into smooth muscle cells and endothelial cells, indicative of their possible pluripotency. When injected into the border zone of hearts with new infarcts, cardiac c-*kit*$^+$ cells led to bands of regenerating myocardium, contributed to endothelium and vascular smooth muscle, and improved the function of the heart (12). Although reported to be present in human myocardium as well (231, 233), significant regeneration is not observed following myocardial infarction. Therefore, either the endogenous c-*kit* positive cardiac stem cells are not responsive to local growth signals or are unable to migrate and differentiate in response to infarction.

There is an additional independent adult mouse heart-derived cardiac progenitor cell expressing stem cell antigen 1 (Sca-1$^+$) (168). On the basis of immunophenotyping, these cells appear to be distinct from c-*kit*$^+$ stem cells in the heart. When subjected to 5-azacytidine treatment, cardiac Sca-1$^+$ cells activate several cardiac-specific genes in vitro. When injected intravenously into mice 6 h after myocardial infarction, engrafted Sca-1$^+$ donor cells expressed cardiac markers, suggesting that they differentiate into cardiac myocytes in vivo. Approximately one-half of the donor-derived cells fused with host cardiomyocytes and 50% differentiated without fusion. It remains to be determined whether cardiac Sca-1$^+$ cells have restricted developmental potential to only differentiate into cardiomyocytes or whether they will be able to differentiate into other cell types similar to c-*kit*$^+$ cells. Another cardiac-derived subpopulation with progenitor potential, which likely overlaps with the Sca-1$^+$ cells, is a rare population of cells termed side population (SP) cells (147, 178). They were isolated from mouse hearts based on their ability to exclude Hoechst dye, which was shown to be dependent on the expression of the ABCG2 transport protein, a member of the family of ATP-binding cassette (ABC) transporters (147). These cells are present throughout cardiac development, also express Sca-1$^+$, but are rare, and their ability to differentiate into cardiomyocytes and contribute to functional repair of the damaged myocardium needs to be fully evaluated.

Most recently, it was demonstrated that a subpopulation of cells in the anterior pharynx expresses the homeobox gene *islet-1* (*isl1*) (27). During development, *isl1*$^+$ cells contribute to formation of the outflow tract, the atria, and the right ventricle (27). Expression of *isl1* is lost when these cells differentiate into cardiomyocytes. Some *isl1*$^+$ cells have been identified in the mature hearts of newborn rodents and humans where they remain undifferentiated (121). These cells fail to express Sca-1, CD31, or c-kit, though they express Nkx 2.5 and GATA4. It was demonstrated that these cells could differentiate into cardiomyocytes both in vivo and in vitro (121). It is not known whether *isl1*$^+$ cells exist in the adult heart beyond the early postnatal period. Moreover, the capacity of isl1$^+$ cardiac progenitors to home to damaged myocardial tissue and form functional myocytes remains to be determined.

These data argue that the postnatal heart has one or more populations of resident stem or progenitor cells that might be utilized to regenerate the heart after injury. Since it is unlikely that multiple stem cells exist, it will be important in the future to determine the hierarchy of these various cells. It is also apparent that even if a cardiac stem cell exists, these cells by themselves are not capable of mounting a robust response to repopulate damaged myocardium as is seen in the newt and zebrafish. Clinical use of these progenitor cells will require a better understanding of the signals involved in the activation of their proliferation and migration to the site of injury or, alternatively, isolating and expanding them in vitro before reintroducing them into the damaged myocardium.

VI. POTENTIAL FOR THERAPEUTIC MANIPULATION OF CARDIAC MYOCYTE CELL CYCLE

An alternative approach to the use of stem cells for cardiac repair is to reactivate the proliferative potential of existing differentiated cardiac myocytes as is seen in lower vertebrates. Investigators have taken a number of approaches to genetically manipulate key cell cycle regulators to promote cell cycle progression. Since proliferating cardiac myocytes express high levels and activity of cell cycle promoting factors such as cyclins D1, E, A and B, Cdk2, Cdk4/6, and Cdc2 as well as E2F family members and low levels of the Cdk inhibitors p21 and p27 (22, 180), attempts to induce cardiomyocyte proliferation have focused on overexpressing cell cycle regulatory factors to promote cell cycle progression. A number of studies (summarized in Table 2) have shown that constitutive expression of fundamental cell cycle regulators can stimulate DNA synthesis in cardiomyocytes and in some cases lead to complete genomic replication and karyokinesis, but cytokinesis remains an elusive goal in the adult heart. The earliest studies overexpressed viral oncoproteins such as adenovirus E1A and SV40 large T-antigen to override cell cycle checkpoints. Targeted expression of SV40 T-antigen was sufficient to induce sustained cycling of cardiomyocytes both in embryonic and adult heart (71, 203). De novo expression of E1A or its downstream effector, E2F-1, although activating DNA synthesis resulted in widespread apoptosis that limits its usefulness as a regeneration strategy (113).

Inducible activation of c-Myc in adult myocardium resulted in cell cycle reentry and nuclear mitosis without evidence of apoptosis, but whether cytokinesis occurred is questionable (100). In another study, transgenic hearts that expressed high levels of Cdk2 mRNA showed significantly increased levels of Cdk4 and cyclins A, D3, and E and augmented DNA synthesis in the adult animal but limited ongoing DNA synthesis (136). Overexpression of Cyc D1, D2, or D3 in transgenic mice was sufficient to stimulate DNA synthesis in adult myocardium under baseline conditions, although differences in biological activity were uncovered after cardiac injury (175). In mice expressing cyclin D1 or D3, there was a reduction in the level of cardiomyocyte DNA synthesis after injury. In contrast, cardiac injury in mice expressing cyclin D2 did not alter cardiomyocyte cell cycle. Because infarct size was similar at 7 days but decreased by ~30% in CycD2 overexpressing mice at 150 days compared with nontransgenic littermates, the authors concluded that regeneration had occurred. It should be noted, however, that given at least a subset of the cardiac myocytes in the CycD2 hearts had never undergone terminal differentiation, it will be important to determine whether de novo expression of CycD2 in adult myocardium using inducible transgenics or gene therapy has a similar effect. Elevated DNA synthesis and mitotic index were also demonstrated in cyclin A2 overexpressing transgenic mice (31). This group of investigators also demonstrated that delivery of a cyclin A2 expressing adenoviral vector immediately postinfarct enhanced cardiac function postinjury (241). Recently, another study demonstrated that treatment with FGF1 in combination with a p38 MAPK inhibitor after myocardial injury could improve cardiac function, reduce scarring, and induce mitosis in cardiac myocytes (61). This and the other examples outlined above are very promising approaches to regenerate myocardium; however, the results should be interpreted with caution. In many cases, the effects of the genetic or therapeutic maneuver on infarct size were not determined or, alternatively, a reduction in infarct size was reported. Thus it is not clear how much of the beneficial effects of the therapy were related to a reduction in infarct size versus true regeneration. Likewise, it will be important in future studies to document evidence of true cytokinesis where regeneration is thought to have occurred and either delay therapy with potential therapeutics to a time after myocardial infarction where infarct size is not affected or utilize inducible transgenics.

VII. SUMMARY

Investigators and clinicians have been faced with the therapeutic challenge that, regardless of your belief on the proliferative potential of the adult cardiac myocyte or the presence of stem cells capable of differentiating into cardiac myocytes, the heart has little, if any, potential for regeneration after injury. This limits the adaptive response to the increased hemodynamic stress following cardiac injury primarily to cardiac hypertrophy and leads to alterations in the expression of a panel of genes that have been proposed to contribute to the eventual deterioration in cardiac function that leads to congestive heart failure. Consequently, investigators searched for strategies to increase myocardial mass after injury through increasing myocyte number versus cell size. The field of cardiac regeneration has exploded since the identification of stem and progenitor cells capable of differentiating into cardiac myocytes. While much research presently focuses on the identification, expansion, and therapeutic use of these cells, results from human studies thus far have been mixed, and the ability of the cells to actually differentiate in cardiac myocytes has been questioned (157).

Nonetheless, it is likely, given the limited proliferative potential of cardiac myocytes or stem cells (12), that an understanding of the regulation of the cardiac cell cycle will be necessary for the field of cardiac regeneration to continue to advance regardless of whether it is to therapeutically reactivate cell cycle progression in exist-

TABLE 2. *Genetic manipulations affecting cardiomyocyte proliferation/DNA synthesis*

Gene	Modifications Done	Observed Phenotype	Reference Nos.
	Cell cycle mediators		
Cyc D1/D2/D3	Transgenic mouse under the control of α-MHC promoter	Increased multinucleation and DNA synthesis in adult cardiomyocytes	26,175,214
Cyc A2	Transgenic mouse under the control of α-MHC promoter	Postnatal hyperplasia but with no evidence of cytokinesis	31
Cyc B1/Cdc2	Adenoviral transfer in neonatal and adult rat cardiomyocytes	Increased cell number	122
Cdk2	Transgenic mouse under the control of α-MHC promoter	Increased DNA and PCNA synthesis in adult cardiomyocytes	136
	Cell cycle inhibitors		
p27	Knockout mouse model	Increased heart size, cardiomyocyte number, DNA and PCNA synthesis in adult cardiomyocytes	181
p53	Adenoviral transfer in neonatal rat cardiomyocytes	Activates mitochondrial death pathway and promotes apoptosis	186
p38α MAPK	Conditional knockout mouse model using Cre/Lox system	Promotes complete division in adult cardiomyocytes	62
RB/p130	Double-knockout mouse model using Cre/Lox system	Increased heart size, BrdU, and His H3 positive adult cardiomyocytes	142
TSC2	Homozygous mutation at TSC2 locus in Eker rat embryos	Sustained proliferation of cardiomyocytes from mutant embryos in vitro	171
TSC2	Targeted expression of a dominant-negative TSC2 cDNA under the control of α-MHC promoter	Increased DNA synthesis in isoproterenol induced hypertrophy in adult cardiomyocytes	174
	Transcription factors		
E2F1	Adenoviral transfer in adult rat cardiomyocytes	Increased DNA synthesis and apoptosis	2
E2F1/in p53 KO	Adenoviral transfer in adult mouse cardiomyocytes	Increased DNA synthesis and apoptosis	2
E2F2/E2F4	Adenoviral transfer in neonatal mouse cardiomyocytes	Induces S-phase entry	59
c-Myc	Transgenic mouse under the control of α-MHC promoter	Hyperplastic growth of heart during embryonic stage and accelerated hypertrophic growth during neonatal stage	100,141
c-Myc	Transgenic mouse under the control of α-MHC promoter	Increased cell cycle reentry with increase in multinucleation and ploidy G_1-S-G_2M	245
Jumonji	Knockout mouse model	Increase in the number of mitotic cardiomyocytes	104
	Growth factors		
FGF-2	DNA transfection in neonatal rat cardiomyocyte cultures	Increased DNA synthesis and cell number	169
FGFR-1	DNA transfection in neonatal rat cardiomyocyte cultures	Increased DNA synthesis and cell number	204
FGFR-1/FGFR-2	Double-knockout mouse model using Cre/Lox system	Severe hypoplasia, enlarged atria, and thinning of the ventricular wall	19
IGF-I	Transgenic mouse under the control of α-MHC promoter	Increased number of cardiomyocytes	187
TGF-βRIIΔKD/FGF-2	Adenoviral transfer in neonatal rat cardiomyocytes	Increased DNA synthesis	204
TGF-β type-IR-ALK5	Transgenic mouse under the control of α-MHC promoter	Linear, dilated, hypoplastic heart tube	30
	Tumor virus oncoproteins		
SV40-TAg	Transgenic mouse under the control of ANF promoter	Mice developed atrial tumors and arrhythmias	217
SV40-TAg	Transgenic mouse under the control of protamine promoter	Mice developed cardiac rhabdomyosarcomas	233
SV40-TAg	Transgenic mouse under the control of α-MHC promoter	Developed atrial and ventricular tumors	111
SV40-TAg	Adenoviral transfer in neonatal rat cardiomyocytes	Increase in the number of mitotic cardiomyocytes	203
Temperature-sensitive (tsA58) mutant of SV40-TAg	Retroviral delivery in adult rat cardiomyocytes	Increase in the number of mitotic cardiomyocytes	107
E1A	Adenoviral transfer in embryonic rat cardiomyocytes	Increased DNA synthesis and apoptosis	139
E1A/E1B	Adenoviral transfer in neonatal rat cardiomyocytes	Increased DNA synthesis without apoptosis	113
E1A	Adenoviral transfer in embryonic rat cardiomyocytes	Increased DNA synthesis and apoptosis	194
E1A/E1B and hCDC5	Adenoviral transfer in neonatal rat cardiomyocytes	Promotes G_2/M progression and complete division	91

MAPK, mitogen-activated protein kinase; TSC, tuberous sclerosis complex; KO, knockout; FGF, fibroblast growth factor; IGF-I, insulin-like growth factor I; TGF, transforming growth factor; SV40, simian virus 40; MHC, myosin heavy chain; ANF, atrial natriuretic factor; BrdU, bromodeoxyuridine.

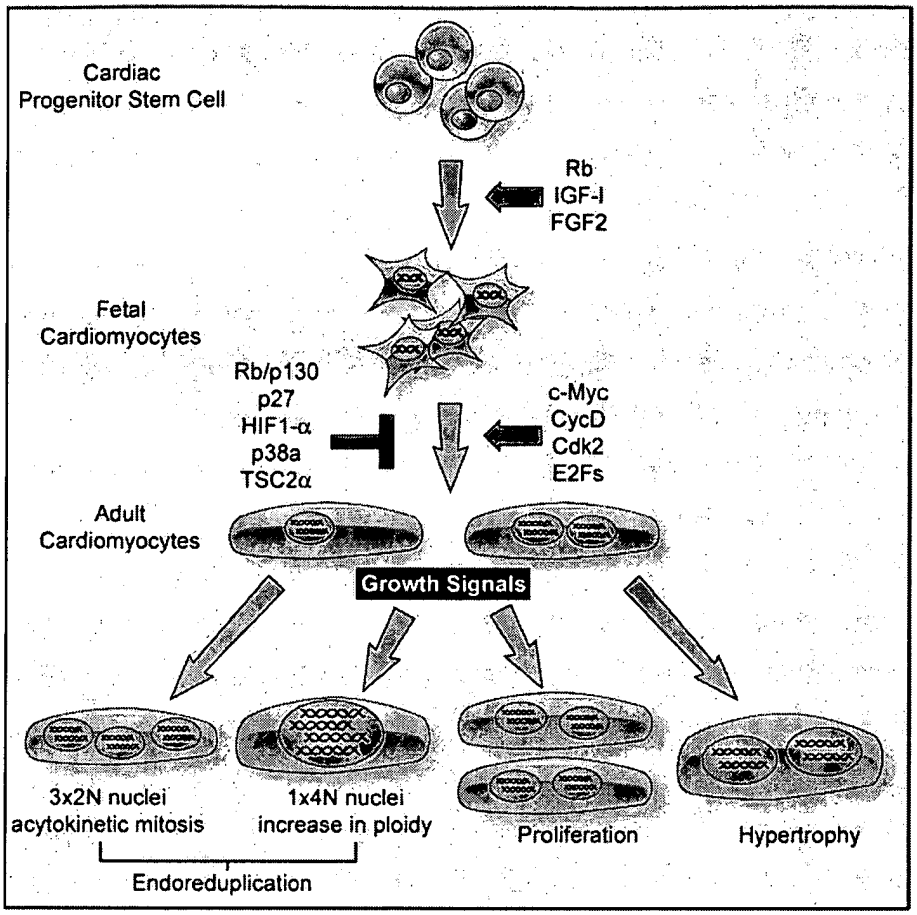

FIG. 2. Control of cardiac cell cycle. Schematic representation of the factors influencing cardiac myocyte cell cycle progression and proliferation at different developmental stages. The potential outcomes of growth factor stimulation of adult cardiac myocytes, namely, proliferation, endoreduplication, and hypertrophy in cardiac myocytes, are also shown.

ing cardiac myocytes or to establish methodologies to expand cardiac stem cells without losing their differentiation potential. This review has attempted to summarize the dramatic advances that have been made in our understanding of cardiac myocyte cell cycle control in recent years (Fig. 2). Despite these advances, major hurdles remain before manipulating the cardiac cell cycle could be used therapeutically, such as lack of cytokinesis in adult myocytes and the challenge that maneuvers to induce cell cycle reactivation in adult myocytes are often accompanied by unwanted cell death.

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